

EFFECT OF RNA INTERFERENCE ON *MYB* TRANSCRIPTS DURING
FLOWER DEVELOPMENT IN *DENDROBIUM* ORCHID

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ABSTRACT

RNA interference is currently a widely used technique for gene silencing studies in several plant and animal systems. In this study a rapid and inexpensive RNAi technique was used for manipulation of endogenous gene expression in *Dendrobium* orchids using large quantities of dsRNA which were synthesized in HT115 *E. coli*. A partial cDNA clone of the *MYB9* gene was isolated from *Dendrobium* Sonia flowers and this was confirmed by sequencing and local alignment analysis using BLAST. The *MYB9* gene sequence was cloned into a bacterial expression vector in forward and reverse orientations and transformed into HT115 *E. coli* strain where induction of bacterial RNA expression was performed by addition of IPTG. RNA produced within the two transformed clones of HT115 *E. coli* cells was extracted and then annealed to form a crude bacterial extract containing dsRNA. RNA interference studies on orchid buds were carried out by treatment with the crude bacterial extracts mixed with cellite. The effect of the forward, reverse and dsRNA extracts were observed on orchid buds that flowered. Transcripts of the *MYB9* gene were successfully reduced in this experiment however this had no visible effect on flower colour. This study has shown that HT115 *E. coli* can be successfully used to produce large quantities of dsRNA in a short period of time at relatively low cost. The mixture of RNA was able to reduce expression of the target mRNA in *Dendrobium* flower petals, demonstrating that this might be a useful tool for orchid research and biotechnology.

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LIST OF SYMBOLS AND ABBREVIATIONS

AS- acetosyringone	HR- hypersensitive response
DNA-deoxyribonucleic acid	GA- gibberellin
T-DNA- transfer DNA	bp – base pair
cDNA- complementary DNA	IPTG- isopropyl- β -D-thiogalactopyranoside
RNA-ribonucleic acid	TBE- Tris borate EDTA
RNAi- RNA interference	TE- Tris-EDTA
dsRNA- double stranded RNA	EDTA- ethylenediaminetetraacetic acid
ssRNA- single stranded RNA	HCL- hydrochloric acid
siRNA- small interfering RNA	SDS- sodium dodecylsulphate
mRNA- messenger RNA	OD- absorbance
rRNA- ribosomal RNA	OD ₅₉₅ - Absorbance at wavelength 595nm
RNase- ribonucleas	mg- milligram
PTGS- post transcriptional gene silencing	ul – microliter
DCL- dicer like	ug – microgram
RISC- RNA induced silencing complex	M- molar
hpRNA- hair pin RNA	mM- millimolar
PPT-phosphinothricin	rpm- revolutions per minute
HDGS-homology dependent gene silencing	dH ₂ O- distilled water
RdDM-RNA directed DNA methylation	ng – nanograms
UV- ultraviolet	g – grams
VIGS- virus induce gene silencing	(x) g – times gravity (relative centrifugal force)
PLB- protocorm like body	
ABA- abscisic acid	

CHAPTER 1: INTRODUCTION

Orchids belong to the largest family of flowering plants, the *Orchidaceae*. There are an estimated 35000 species found throughout the world in diverse habitats (Hsieh and Weng, 2005). Research into developing new varieties of orchids has intensified in the last few years in order to meet the high demand for orchids in the market. Orchids are part of a growing industry that is economically important to Malaysia and new orchid varieties could potentially increase exports to other countries. In order to find inexpensive, fast and efficient methods to develop new orchid varieties with desirable characteristics, biotechnology techniques could be applied.

The cut flower industry in Malaysia is showing tremendous growth, especially in the last ten years, with flowers and ornamental plants being exported to other countries. Orchid growing is a multi-million Ringgit industry with approximately 24.3 million stalks of orchid cut flowers produced in the year 2000, of which *Dendrobium* species topped the list with 13.1 million stalks being produced (Janna *et al*, 2006). The most popular orchid types cultivated were *Dendrobium* (11.17%), *Aranda* (8%), *Oncidium* (5%) and *Mokara* (3.5%). Together these 4 genera produced 97% of the countries orchid cut flowers in 1994 (Lim *et al*, 1998). There is an increasing demand for orchids throughout the world especially those with variations in flower pigmentation, flower shape or size, longer vase life and better fragrance. Other desirable traits in orchids include resistance to disease and stress, early flowering and increasing the number of flowers produced per inflorescence (Stephen and Chin, 2005).

In the past conventional orchid breeding methods were widely used to select offspring with desirable characteristics. Classic breeding techniques mainly involved crossing between or within related species and selection of offspring or searching for mutants with desirable characteristics such as unique flower colour and shape. The drawbacks of these classic breeding methods are that they are time consuming and tedious, since orchids have long reproductive cycles (several years) and slow seed maturation. Although conventional breeding methods have been used in the production and improvement of several commercially available orchids, the available gene pool for new traits is limited and since the selection of traits in the offspring is based on the genetic characteristics of the two parents, this complicates and limits controlled breeding (Zuker *et al*, 1998). In the past few years several new techniques have evolved to improve orchids and other ornamental plants.

New biotechnological approaches and molecular breeding techniques have been used to manipulate orchid traits and have enabled the broadening of the gene pool for a given species. More efficient transformation techniques have come into use, which could meet the demand for unique traits not just in orchids but in other ornamentals as well (Yang *et al*, 1999). Some new technologies being used to introduce foreign genes into orchids include particle bombardment and *Agrobacterium* mediated transformation techniques (Yu and Xu, 2007). There are several other potential genes that can be used for genetic engineering in orchids and new methods of genetic engineering are being developed to improve orchids.

RNA interference is a technique that uses dsRNA for gene silencing and has potential application to orchid gene manipulation. It could be used to produce new orchid varieties in a short time at lower costs compared to other genetic engineering techniques. This technique can be used to silence certain genes that are involved in different aspects of flower development such as, flower morphology, flower colour, fragrance, flowering time, number of flowers per inflorescence and this factor makes RNA interference an important technique to explore for development of new orchid varieties. Since this technique can be applied to orchids that are about to flower it could be less time consuming and tedious as compared to other methods of orchid transformation.

1.0 Objectives

- 1) To design and make RNA interference constructs for the knock down of expression of specific *MYB9* transcription factors.
- 2) To study the effect of *MYB9* gene silencing on *Dendrobium* flower colour using RNA interference

CHAPTER 2: LITERATURE REVIEW

2.0 Orchids

Approximately 800 species of orchids covering 120 genera are thought to be indigenous to Peninsular Malaysia (Teo, 1995), however this figure has surely increased as it is estimated that around 800 new orchid species are discovered each year around the world. Orchids can be found in diverse environments and this in turn relates to their vegetative morphology. Terrestrial orchids have their roots in the soil and usually they predominate in temperate regions such as Europe, North America, Argentina, southern Australia, Asia and Africa (Bechtel *et al*, 1992). Epiphytic orchids are anchored on branches and on the canopy of forest trees. They basically live on other plants without harming them and can be found in the tropics of Asia, America, Australasia and Africa. Another group called lithophytic orchids grows on rocks. Epiphytic orchids can have either sympodial or monopodial growth forms.

2.0.1 Classification of orchids

The scientific classification of orchids follows the classification system developed by Robert Louis Dressler who was an orchid specialist. He published a book called “The Orchids: Natural history and classification”. This book is widely accepted by botanists and it classifies orchids based on morphology, and other characters such as anther configuration and pollinarium structure. The book was first published in 1981 and then modified several times, the latest version was in 1993 and this is currently being used to classify orchids. Orchids belong to the order *Asparagales* and the family *Orchidaceae*. According to Dressler’s classification the *Orchidaceae* are divided into 5 subfamilies namely the *Apostasioideae*, the *Cypripedioideae*, the *Orchidoidea*, the *Epidendroideae* and the *Spiranθοideae* (Bechtel *et al*, 1992).

2.0.2 *Dendrobium* orchids

Dendrobium orchids belong to the subfamily *Epidendroideae* and genus *Dendrobium* which contains approximately 1200 species or more. They are either epiphytic (grow on trees) or lithophytic (grow on rocks). The leaves are usually ovate, oblong, lanceolate or linear and positioned alternately along the stem. The inflorescence is racemose with many flowers. The flowers of orchids contain the following parts as shown below in the *Dendrobium* Sonia orchid (Figure 2.1).



Figure 2.1: Different parts of *Dendrobium* Sonia orchid flower

The distinguishing features of orchids are mainly seen in the flowers. They usually have reduced parts, fusion of these parts and a modified or enlarged labellum. Orchid flowers have their parts in whorls of three. The lower most whorls of floral segments consists of three sepals, usually the dorsal sepal could differ in shape and size from the other two lateral sepals (Bechtel *et al*, 1992). In most orchids the sepals look dull and are not brightly coloured like the petals, however there are exceptions with certain orchid species having sepals that look similar to the petals or even more colourful than the petals. The corolla consists of three segments as well, two petals positioned on either side of the dorsal sepals

and the labellum. The petals are usually brightly coloured. The labellum is highly modified and usually larger than the other two petals; it is located at the lower region of the flower and acts as a platform for potential pollinators.

Orchids have resupinate flowers, meaning since the lip lies in the upper region of the bud; the pedicel has to turn through 180° in order to allow the labellum to attain a ventral position. This feature is called resupination (Bechtel *et al*, 1992). In orchids the male and female parts are usually fused into a single structure called the column. This feature distinguishes them from most monocotyledonous flowers. This genus of orchids produces pseudobulbs as shown in *Dendrobium Sonia* (Figure 2.2) which join into a long reed like stem. Pseudobulbs are water storage organs formed by the swelling of internodes in the stem. They can be of different shapes and sizes in different species.

Dendrobium Sonia orchids are a cross between *Dendrobium Caesar* and *Dendrobium Tomie Drake* (a hybrid). The hybrids are generally considered hardier than other genera and they are also fast and easy to grow. The inflorescence of *Dendrobium Sonia* can grow to about 60cm long and bear as many as 15 flowers. The flower itself is about 8.5cm across and 7.5cm tall. The lateral petals are dark reddish purple and white at the base close to the column. The sepals are tinged purple with a white background and show a faint thin band in the middle (Tan and Hew, 1993).

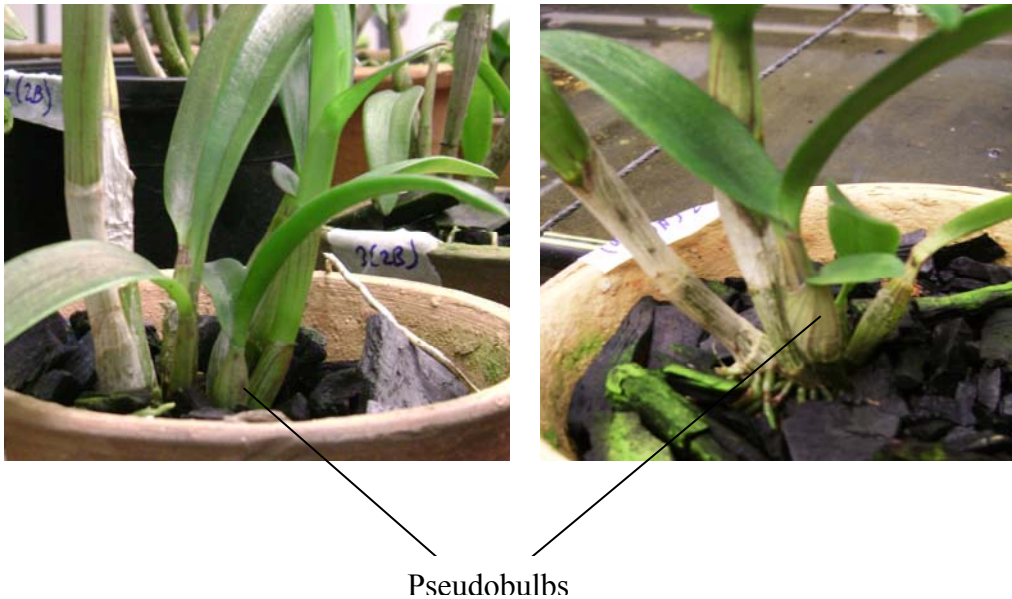


Figure 2.2: *Dendrobium Sonia* pseudobulbs

2.1 Orchid Biotechnology

The genetic transformation of orchids involves the introduction of exogenous genes or genetic material into the plant chromosome. These introduced genes are either expressed or interrupt expression of endogenous genes within the orchid and this could results in transgenic plants with unique traits. Transformation techniques applied to orchids can be quite inefficient and until recently have been applied successfully only to a few orchid genera such as *Dendrobium*. Transformation efficiency was found to be low in certain orchid genera such as *Cymbidium*, *Phalaenopsis*, *Cattleya*, *Brassia* and *Doritaenopsis* (Men *et al*, 2002). In order to develop simple and more efficient transformation techniques factors such as transformation method, starting materials and selection markers have to be identified (Yu and Xu, 2007). The two most frequently used methods of transformation in orchids are particle bombardment and *Agrobacterium* mediated transformation.

2.1.1 Genetic transformation of orchids

Particle bombardment also known as biolistic bombardment or microprojectile bombardment is an effective method to introduce DNA into plant cells that involves coating microprojectiles or microcarriers e.g. gold particles, with DNA and then propel them into plant cells usually through gas acceleration or using a particle gun. Factors that could affect the process include type, amount and size of the microcarriers and acceleration parameters. Particle bombardment is highly efficient in introducing DNA into the plant cells however integration of this DNA into the plant genome is less efficient. Transient expression of the genes occurs in most cases where the DNA enters the cells and is expressed for a short period of time before it is degraded by nucleases (Altpeter *et al*, 2005). Although DNA is propelled into plant cells if it does not integrate into the plant genome transgenic plants will not be produced.

The use of particle bombardment in orchid transformation was first reported in *Dendrobium* and *Vanda* orchid genera. It was the most widely used transformation method for orchids in the past decade (Yu and Xu, 2007). The selection efficiency of particle bombardment is determined by multiple factors. It was found that orchid tissues usually sustain a certain amount of damage after particle bombardment and require a healing period, thus the timing of selection varies among orchid species and directly affects transformation efficiency. For example the protocorms of *Dendrobium* hybrid “Mihua” died when selection was initiated two weeks after particle bombardment but transformation efficiency was higher with selection was delayed up to 3 months after bombardment. However in *D. phalaenopsis* and *D. nobile* selection two days after bombardment resulted in higher transformation efficiency compared to selection delayed up to 30 days after bombardment (Yu and Xu, 2007).

The orchids *D. nobile* and *D. phalaenopsis* were transformed by biolistic bombardment of calli and protocorm like bodies (PLB) using gold particles (0.1µm) coated with plasmid DNA. Selection was done using hygromycin and the highest transformation efficiency was obtained 2 days after bombardment. It was found that in *D. phalaenopsis* a transformation efficiency of approximately 12% was obtained while for *D. nobile* the transformation efficiency was between 0.5% and 4%. Thus transformation efficiency varied among species (Men *et al*, 2002). When selection was delayed by one month no transformants were obtained indicating that late selection resulted in lower transformation efficiency.

The type of plant tissue used for particle bombardment may also affect transformation efficiency. Generally orchid cells have low rate of proliferation and orchid cells in tissue culture produce high amounts of phenolics that become toxic to cells when oxidized. Orchid cells also remain unaffected by tissue culture manipulations making their transformation requirements different from other plant tissue (Yang *et al*, 1999). It has been found that culture of orchid calli is more difficult as they grow slowly and could become necrotic, therefore orchid calli are used in very few studies (Men *et al*, 2002). Selection markers also influence transformation efficiency both in particle bombardment and *Agrobacterium* mediated transformation systems. Other challenges encountered when using particle bombardment include increasing transformation efficiency and reducing frequency of chimerism in transformants. Particle bombardment has been used successfully for orchid transformation to obtain useful traits such as virus resistance, modifying flowering time, manipulating flower morphology and other ornamental qualities (Yang *et al*, 1999).

Agrobacterium-mediated transformation involves transfer of T-DNA from the pathogenic soil bacterium *Agrobacterium tumefaciens* that infects several plant varieties causing crown gall disease. The transfer of T-DNA from *A. tumefaciens* into plant cells is somewhat similar to bacterial conjugation where the T-DNA, which is part of the Ti plasmid, is inserted into the plant genome. The *vir* (virulence) genes which reside on the Ti plasmid are necessary for the transfer of T-DNA into the plant cells. Tumorigenesis occurs due to expression of the *onc* genes present on the T-DNA. Scientists exploited this mechanism of T-DNA integration (natural plant transformation system) to introduce genetically engineered DNA into the T-DNA region (Madigan *et al*, 2003). The process of *Agrobacterium*-mediated transformation involves three basic steps, cloning of genes of interest into the T-DNA, introduction of T-DNA into *Agrobacterium* strains and integration of T-DNA into plant genomes via *Agrobacterium*-mediated infection of host plants (Yu and Xu, 2007). *A. tumefaciens* has a broad host range infecting mostly dicotyledons; however monocotyledons (orchids) and gymnosperms are recalcitrant to *Agrobacterium*-mediated transformation. It was found through biochemical tests that coniferyl alcohol is a potent inducer of *vir* genes present in *Dendrobium* orchids and was found to be essential for *Agrobacterium*-mediated transformation (Men *et al*, 2003).

The success of *Agrobacterium*-mediated transformation is dependent on the concentration of phenolic compounds present in the transformation system and the period of co-cultivation of *A. tumefaciens* with orchid explants (Yu and Xu, 2007). In experiments on *D. nobile* PLBs it was found that the addition of acetosyringone (AS) to the inoculation and co-cultivation media increased the transformation efficiency. Higher transformation efficiency was obtained with a longer co-cultivation period. The inoculation period also affected transformation efficiency. An inoculation period of 30 minutes resulted in a high

transformation efficiency of 18%, however when the inoculation period was increased to 60 minutes the transformation efficiency reduced to 3-4% (Men *et al*, 2003). It has been found that AS greatly increases transformation efficiency not only in *Dendrobium* but also in *Phalaenopsis* and *Oncidium* orchids and when it is added during pre-culture it could increase *vir* gene inducing activity in *A. tumefaciens* thus stimulating the efficiency of *Agrobacterium* infection (Mishiba *et al*, 2005; Liao *et al*, 2003). The optimum conditions for AS of certain concentration to activate the *vir* gene of the Ti plasmid and initiate T-DNA transfer was at pH 5.0-5.5 (Yu and Xu, 2007).

2.2 Selectable markers for transformation systems

Selectable markers are an important part of transformation systems. They are introduced as marker genes into the plant genome and identified in plants that have been putatively transformed through a specific selection agent. The selection markers that are currently used for screening transgenic orchids include antibiotic resistant genes, herbicide resistant genes, pathogen resistant genes and visual reporter genes (Yu and Xu, 2007). Generally sensitivity of plants cells to selectable markers depends on the genotype, physiological conditions, size and type of explants used and tissue culture conditions (Yang *et al*, 1999).

2.2.1 Antibiotic resistant genes as selectable markers

The two most widely used antibiotics for transformation studies in orchids are kanamycin and hygromycin. Kanamycin resistance is through the introduction of the *NPTII* gene (neomycin phosphotransferase) that can phosphorylate aminoglycoside antibiotics such as kanamycin, neomycin, geneticin and paromomycin causing inactivation (Yu and Xu, 2007). Usually kanamycin is used in high concentrations to make selection more stringent and since orchids have low sensitivity to kanamycin, instead of using such high

concentrations (500mg/l) which mean higher costs, alternative selection methods like use of the luciferase firefly gene are coming into use. Since the sensitivity of plant cells depends on the genotype, in a study on *Cymbidium* orchids, the minimum amount of kanamycin required to fully inhibit growth of untransformed cells had to be determined. Different concentrations of kanamycin were used (0, 50, 100, 200, 500mg/l) and 846 PLBs of three *Cymbidium* genotypes were grown on solid media. After 45days it was found that all PLBs grown on media containing 100mg/l kanamycin or higher had died. It was determined that the minimum concentration of kanamycin needed for selection of transformed PLBs was 50mg/l and untransformed PLBs died at this concentration or lower (Yang *et al*, 1999). To avoid the formation of chimeric plants, high concentrations of kanamycin are used and a long exposure time is required. However it has been found that hygromycin is more toxic than kanamycin therefore it can be used to eliminate sensitive cells more quickly.

Hygromycin resistance is through introduction of the *hpt* gene encoding hygromycin phosphotransferase. Hygromycin interferes with protein synthesis in plants and since it is more toxic than kanamycin, it can be used to eliminate untransformed cells quickly, making it more useful in reducing selection time and frequency of chimeric plants. In studies conducted on *Dendrobium*, *Phalaenopsis* and *Oncidium* orchids, concentrations of hygromycin used were between 5-50mg/l (Yu and Xu, 2007; Liao *et al*, 2003; Mishiba *et al*, 2005).

2.2.2 Herbicide selection

The *bar* gene from *Streptomyces hygroscopicus* can be used as a selectable marker which confers resistance to the herbicide phosphinothricin (PPT) and bialaphos which is an alanine derivative of PPT. Bialaphos and PPT have been shown to be effective selective agents for transformation in both dicots and monocots (Knapp *et al*, 2000). The *bar* gene encodes phosphinothricin acetyl transferase that can detoxify PPT and bialaphos through acetylation of free amino acid residues, thus plant cells into which the *bar* gene has been introduced can grow on media containing PPT and bialaphos. In a transformation study conducted on three unrelated orchid genera *Cattleya*, *Brassia*, *Phalaenopsis* and *Doritaenopsis* using the *bar* gene and bialaphos, it was suggested that this transformation system could be applied to any orchid genera that could be manipulated through tissue culture (Knapp *et al*, 2000; Yu and Xu, 2007). Particle bombardment was used to introduce the *bar* gene construct into the PLBs of *Brassia* and *Doritaenopsis* and germinated seeds of *Cattleya*. These were grown on solid media containing bialaphos and after 4 weeks it was observed that 90% of all tissue became necrotic. From 32 putative transformants, 11 *Brassia*, 18 *Doritaenopsis* and 3 *Cattleya* were recovered. This method is advantageous as only 1-3mg/l of bialaphos is sufficient to inhibit growth of untransformed PLBs within 4 weeks of application and this transformation technique could be applied to several orchid genera (Knapp *et al*, 2000).

2.2.3 Visual selection

The luciferase (*LUC*) gene from fireflies has been used successfully as a reporter/marker gene in *Dendrobium* orchid transformation. The method of transformation used was particle bombardment with tungsten (1.3µm) coated with plasmids containing the 35S-*luc*

chimeric gene. The transformed PLBs were identified 4 weeks after particle bombardment by the addition of luciferin that made transformed tissue appear bioluminescent (Chia *et al*, 1994). The transformed tissue was then used to generate transgenic *Dendrobium* plants. Visualization of bioluminescence was through use of low light video microscopy and photon imaging. The technique is expensive due to use of the photon counting imaging technology and this is a disadvantage (Yu and Xu, 2007).

2.2.4 Pathogen resistance selection

A novel transformation technique was applied to *Oncidium* orchids that involved the use of pathogen resistance as a selectable marker for transformed orchid plants. The sweet pepper ferredoxin-like protein (*pflp*) was used as a selection marker and the pathogen *Erwinia carotovora* as a selection agent in *Oncidium* orchids. The plant pathogen *Erwinia carotovora* is well known for causing a disease called soft rot in orchids and *Oncidium* orchids in particular are highly susceptible to this pathogen. The *pflp* gene has been used in other studies where it was discovered that it had antimicrobial properties which were due to production of a peptide that caused a delayed hypersensitive response in non-host plants through release of a harpin proteinaceous elicitor (PSS) that suppresses bacterial growth (Yu and Xu, 2007; You *et al*, 2003).

The *pflp* protein showed antimicrobial activity against *Pseudomonas syringae* and over-expression in rice showed similar activity against *Xanthomonas oryzae*. Thus it was predicted that introduction of the *pflp* protein into *Oncidium* orchids might confer resistance to *E. carotovora* and if this occurred it could be used as a selection marker in orchids. A vector containing the *pflp* gene was transformed into PLBs of *Oncidium* orchids through *Agrobacterium*-mediated transformation. Selection was through infection with *E.*

carotovora and PLBs that were successfully transformed showed enhanced resistance to *E. carotovora* infection due to expression of the *pflp* gene while untransformed PLBs died. Pathogen resistance selection is advantageous as it allows for selection of putative transformants in 2 weeks, thus it is considered faster, less costly and causes fewer side effects to plant development unlike the inhibitory effects observed when antibiotics or herbicides are used (Yu and Xu, 2007; You *et al*, 2003).

2.3 MYB genes

MYB genes are a family of transcription regulators in eukaryotes involved in a variety of biological functions. The *MYB* gene was first discovered in the *v-Myb* oncogene of an avian myeloblastosis virus. Then later other members of the *MYB* gene family were identified in diverse plants and animals. All *MYB* proteins have a common structural characteristic that is the DNA binding domain (*MYB* domain) that is conserved in animals, plants and yeast (Wu *et al*, 2003).

The *MYB* domain consists of 52 amino acids that bind to DNA in a sequence specific manner. It consists of one to three imperfect repeats, *R1*, *R2* and *R3*, however *R2* and *R3* are sufficient for sequence specific DNA binding (Figure 2.3 below). These imperfect repeats adopt a helix-helix-turn-helix conformation to intercalate with the major groove of the target DNA. Vertebrates usually have very few *MYB* genes and three genes in particular *c-MYB*, *a-MYB* and *b-MYB* have been found in higher vertebrates. These all contain three imperfect repeats *R1*, *R2* and *R3* (Figure 2.3, *c-MYB*). However, plants have a larger number of *MYB* genes compared to animals and usually have only *R2* and *R3* repeats which also correspond to the *R2* and *R3* repeats of vertebrates (Fig 2.3, *ZmMYBC1* from maize). For example *Arabidopsis* has 125 *R2R3-MYB* genes.

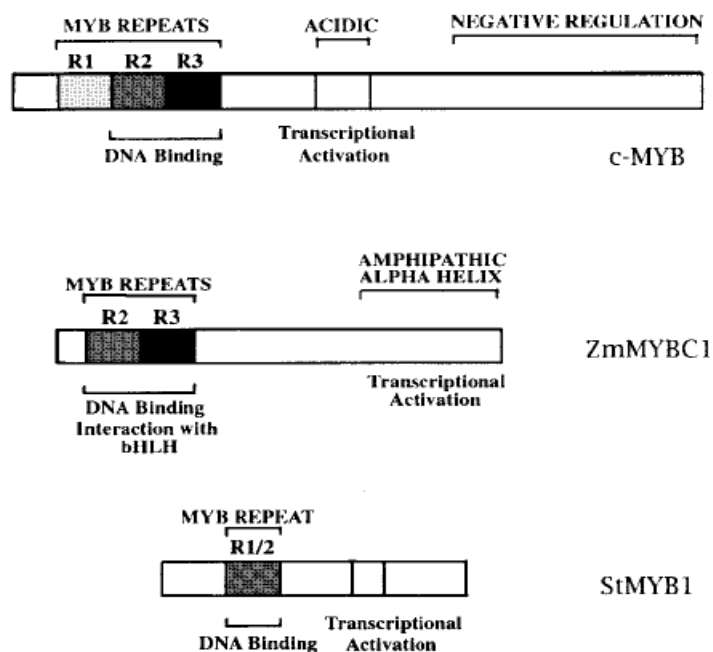


Figure 2.3: Schematic showing functional domains of prototypic *MYB* proteins; three *MYB* repeats (*c-MYB*), two *MYB* repeats (*Cl*) and one *MYB* domain (*StMYB1*) (Jin and Martin, 1999)

Most plant *MYB* genes belong to the *R2R3-MYB* family. Since the *R2R3-MYB* gene family in plants is so diverse, *MYB* genes have diverse functions as well (Table 2.1). Plant *MYB* genes are usually involved in plant specific processes such as regulation of many aspects of plant development including metabolism, hormone signaling and cell shape (Martin and Paz-Ares, 1997). Single *MYB* proteins function primarily as transcription factors however *MYB* genes are also involved in control of secondary metabolism and response to secondary metabolites in plants. Most *MYB* genes are positive regulators of transcription; however a few can also function as negative regulators.

Table 2.1

List of plant MYB-related genes for which function has been assigned. The subgroup for the DNA-binding domain of the R2R3 gene is also listed according to Romero *et al.* (1998)

MYB genes	Biological functions	Species	R2R3 subgroup
One-repeat Myb			
StMYB1	Unknown	<i>Solanum tuberosum</i>	
LHY	Circadian clock regulation, flowering time	<i>Arabidopsis thaliana</i>	
CCA1	Phytochrome & circadian regulation	<i>Arabidopsis thaliana</i>	
PcMYB1	Light-dependent activation	<i>Petroselinum crispum</i>	
CPC1	Epidermal cell differentiation, root hairs	<i>Arabidopsis thaliana</i>	
BPF1	Telomeric DNA binding protein	<i>Petroselinum crispum</i>	
IBP1	Telomeric DNA binding protein	<i>Zea mays</i>	
R2R3 Myb	<i>Phenylpropanoid metabolism</i>		
ZmMYBC1	Anthocyanin	<i>Zea mays</i>	Subgroup C
ZmMYBPL	Anthocyanin	<i>Zea mays</i>	Subgroup C
ZmMYB1	Anthocyanin	<i>Zea mays</i>	Subgroup C
ZmMYB38	Inhibition of C1-mediated activation	<i>Zea mays</i>	Subgroup C
PhMYBAN2	Anthocyanin	<i>Petunia hybrida</i>	Subgroup C
PhMYB3	Anthocyanin	<i>Petunia hybrida</i>	Subgroup C
AmMYB305, 340	Anthocyanin and flavonol	<i>Antirrhinum majus</i>	Subgroup C
PsMYB26	Phenylpropanoid regulation	<i>Pisum sativum</i>	Subgroup C
ZmMYBP	Phlobaphene	<i>Zea mays</i>	Subgroup C
AmMYB308, 330	Phenolic acid	<i>Antirrhinum majus</i>	Subgroup C
	<i>Development</i>		
AtMYBGL1	Trichome development	<i>Arabidopsis thaliana</i>	Subgroup C
AmMYBMIXTA	Conical cell development	<i>Antirrhinum majus</i>	Subgroup C
PhMYB1	Conical cell development	<i>Petunia hybrida</i>	Subgroup C
CotMYBA	Trichome development	<i>Gossypium hirsutum</i>	Subgroup C
AmMYBPHAN	Dorsoventral determination & growth	<i>Antirrhinum majus</i>	AmMYBPHAN subgroup
ZmMYBRS2	PHAN-like, repress <i>knox</i> expression	<i>Zea mays</i>	AmMYBPHAN subgroup
AtMYB13	Shoot morphogenesis	<i>Arabidopsis thaliana</i>	Subgroup C
AtMYB103	Expressed in developing anthers	<i>Arabidopsis thaliana</i>	Subgroup C
	<i>Signal transduction</i>		
GAMYB	Gibberellin response	<i>Hordeum vulgare</i>	Subgroup B
AtMYB2	Dehydration and ABA regulation	<i>Arabidopsis thaliana</i>	Subgroup C
ATR1	Tryptophan biosynthesis	<i>Arabidopsis thaliana</i>	Subgroup C
Cpm5, Cpm7, Cpm10	Dehydration and ABA response	<i>Craterostigma plantagineum</i>	
	<i>Plant disease resistance</i>		
NtMYB1	TMV, SA-inducible	<i>Nicotiana tabacum</i>	
	<i>Cell division</i>		
AtCDC5	Cell cycle regulation	<i>Arabidopsis thaliana</i>	
R1R2R3 Myb			
MSA-binding proteins	Regulation of B-type cyclin genes	<i>Nicotiana tabacum</i>	
AtF4D11.7	Unknown	<i>Arabidopsis thaliana</i>	
AtF6N23.19	Unknown	<i>Arabidopsis thaliana</i>	

2.3.1 Role of *MYB* genes

It has been found that plants *MYB* genes have very distinct and varied functions; however some also have overlapping functions. *MYB* proteins are of particular importance in transcriptional regulation in plants and their functions include regulation of secondary metabolism, control of cellular morphogenesis, in signal transduction pathways responding to plant growth regulators, regulation of meristem formation and the cell cycle (Jin and Martin, 1999; Martin and Paz-Ares, 1997). Structurally similar *MYB* proteins have significant difference in function in different species and even within the same organism.

When *MYB* proteins involved in anthocyanin biosynthesis are compared e.g. from maize *ZmMYBC1* and *ZmMYBPL*, from *Petunia PhMYBN2* and from *Antirrhinum Am-MYBROSEA*, it was found that the proteins of these genes are structurally similar or related in their DNA binding domains and C-terminal sequences. Therefore it can be predicted that these genes are structurally and functionally homologous, however this is not the case. It was found that *ZmMYBC1*, *ZmMYBPL* and *PhMYBN2* regulate different target genes in maize and *Petunia*. In maize *C1* mutants show reduced expression of all structural gene of anthocyanin biosynthesis but in *Petunia AN2* mutants are not affected in their expression of structural genes. *Rosea* mutants from *Antirrhinum* showed reduced expression of different sets of structural genes to *C1* and *AN2* mutants. This shows that although these genes are structurally related and their effect was similar (loss of gene function), the function of each *MYB* protein is not homologous (Jin and Martin, 1999; Martin and Paz-Ares, 1997). It was also found that *MYB* genes are not redundant in their functions even within a single species. For example in maize the genes *ZmMYBC1* and *ZmMYBPL* have the same function to activate transcription of genes controlling anthocyanin biosynthesis, however *ZmMYBC1* works in the aleurone and tissues of the flower while *ZmMYBPL* works in

vegetative plant tissues. This shows that paralogous genes have different expression patterns (Jin and Martin, 1999).

2.3.1.1 MYB genes involved in Phenylpropanoid metabolism

Phenylpropanoid metabolism is a form of secondary metabolism in plants responsible for production of plant pigments. *MYB* genes in plants play an important role in the control of phenylpropanoid metabolism. This involves modification of compounds derived from phenylalanine (Wu *et al*, 2003). There are several examples in which *R2R3 MYB* genes have been shown to play a role in control and regulation of phenylpropanoid metabolism in maize, *Arabidopsis thaliana*, *Antirrhinum majus*, *Petunia*, Tobacco and several other plants. In maize which has more than 80 *R2R3 MYB* genes, the *ZmMYBC1* gene that encodes the *CI* protein has been found to activate transcription of genes that encode enzymes involved in anthocyanin biosynthesis in the aleurone of the maize seeds (Rabinowicz *et al*, 1999). The *ZmMYBPL* gene encodes the *PL* protein that controls anthocyanin biosynthesis in the maize plant including stems and leaves by interacting with members of the R-protein family to activate anthocyanin biosynthetic gene expression. The *ZmMYB1* gene can activate one structural gene involved in anthocyanin biosynthesis while the *ZmMYB38* gene inhibits *CI* mediated activation of the same promoter (Martin and Paz-Ares, 1997).

In *Petunia* which has about 40 *R2R3 MYB* genes, the *PhMYBN2* gene encodes the *AN2* protein that is required for anthocyanin production. Another *Petunia* gene *PhMYB3* which is expressed in the petal epidermis can activate expression of a gene encoding chalcone synthase. In *Arabidopsis thaliana* 126 *R2R3 MYB* genes have been described and it was found that the *MYB12* gene is a flavanol specific regulator of phenylpropanoid

metabolism; it is involved in activation of flavanoid biosynthesis through transcriptional regulation of chalcone synthase and flavonol synthase genes (Mehrtens *et al*, 2005). The *AmMYB305* gene from *Antirrhinum majus* encodes the *MYB305* protein that was found to be involved in activation of phenylpropanoid biosynthetic genes in flowers. *MYB305* and its orthologue in tobacco were found to activate a gene that encodes phenylalanine ammonia lyase which is the first enzyme produced in the phenylpropanoid pathway (Sablowski *et al*, 1994; Martin and Paz-Ares, 1997). In tobacco *NtMYBAS1* was found to activate two different phenylalanine ammonia lyase promoters in the tobacco leaf protoplasts known as *PALA* and *gPAL1*. *NtMYBAS1* was also shown to be an anther specific transcription factor which is a positive regulator of *gPAL1* expression and phenylpropanoid synthesis in saprophytic tissues of the anther (Yang *et al*, 2001). There are many other examples of *MYB* genes involved in phenylpropanoid metabolism, however most *MYB* genes remain uncharacterized and no function has been assigned to these yet therefore further studies into *R2R3 MYB* genes will greatly increase our knowledge of their function in plants.

2.3.1.2 *MYB* genes involved in cell shape

Another role of *MYB* genes is in control of cell shape. This has been observed in *Antirrhinum* and *Petunia* where the *MIXTA* gene and *PhMYB1* gene respectively are essential for conical form of petal epidermal cells (Martin and Paz-Ares, 1997). *AmMYBMX* or *MIXTA* is required for conical-papillate cell formation in the petal epidermis and *PhMYB1* has been shown to have a role in development of conical-papillate petal cells. Another gene from *A. majus*, *AmMYBML1* is involved in development of conical-papillate cells, petal trichome differentiation and formation of the hinge of the ventral petals (Jaffe *et al*, 2007). The gene *AmMYBML3* (*Antirrhinum majus MYB MIXTA*-

LIKE 3), is expressed in outgrowing epidermal cells including trichomes, stigmatic papillae and petal conical-papillate cells. The *AmMYBML3* protein was found to alter epidermal development through acting with other transcriptional activators to enhance cellular outgrowth from the epidermis of aerial organs. Therefore it was suggested that *AmMYBML3* is a weak enhancer of cellular differentiation induced by *AmMYBMX* in petal epidermal cells (Jaffe *et al*, 2007). The gene *GLABROUS1 (GL1)* in *A. thaliana* is required for trichome development and differentiation in the leaves and stem. It is also required for initial expansion of trichome cells and when mutated it gives rise to outgrowth instead of branched trichomes (Martin and Paz-Ares, 1997). In another study it was found that the *WEREWOLF (WER)* gene in *A. thaliana* is required to specify the non-hair cell type in root epidermal cells and the non-stomatal cell type in the hypocotyls (Lee and Schiefelbein, 2001). In a study on cotton it was found that the *GaMYB2* gene may be a regulator of cotton fiber development (Wang *et al*, 2004).

2.3.1.3 MYB genes involved in hormonal response

MYB genes play a role in hormonal responses during seed development and germination. In barley the *GAMYB* gene is a transcriptional activator of the pI α -amylase promoter which is responsible for α -amylase gene expression in aleurone cells during germination. Therefore *GAMYB* expression which is induced by gibberellin (GA), encodes a *MYB* related protein that is involved in GA-regulated gene expression in barley aleurone layers and it was postulated that this protein is related to the GA response pathway leading to α -amylase gene expression in aleurone cells (Gubler *et al*, 1995). The plant hormone abscisic acid (ABA) can induce expression of the *AtMYB2* gene from *A. thaliana* which is usually expressed in response to dehydration or salt stress and in maize it was found that the *C1* gene is also ABA responsive and involved in formation of anthocyanins in the kernels.

2.3.1.4 MYB genes involved in plant disease resistance

An *R2R3 MYB* gene *AtMYB30* from *A. thaliana* has been discovered; that acts as a positive regulator of hypersensitive cell death in plants in response to pathogen attack. The hypersensitive response (HR) is programmed cell death due to pathogen resistance in plants. It was found that the *AtMYB30* gene was specifically and rapidly expressed during interactions between *A. thaliana* and bacterial pathogens. It was also demonstrated through analysis of transgenic plants expressing sense and antisense *AtMYB30* transcript that over expression of *AtMYB30* in *A. thaliana* and Tobacco accelerates and intensifies the HR to different avirulent bacterial pathogens, causes HR like responses to virulent strains and increases resistance to different bacterial pathogens (Vailleau *et al*, 2002).

2.4 Phenylpropanoid metabolism/ Flavonoid biosynthesis

Flower colour is due to the presence of three main pigments, the flavonoids, the carotenoids and the betalains. Flavonoids are the most common and abundant pigment of the three and contribute to a range of colours from yellow to red to blue (Tanaka *et al*, 2005). Phenylpropanoid metabolism is one of the major types of secondary metabolism in higher plants and it is responsible for the formation of major plant pigments such as the anthocyanins, flavonols, flavones, aurones and isoflavonoids (Figure 2.4). More than 8000 different flavonoids have been identified in higher plants and they can be divided into seven major subgroups, the chalcones, flavones, flavonols, flavandiols, anthocyanins, proanthocyanidins (tannins) and the aurones (Winkel-shirley, 2001). There are also other minor subgroups of flavonoids such as isoflavonoids, phlobaphenes and stilbenes which are synthesized by specific plants. Flavonoids have many diverse functions in higher plants. They are responsible for the formation of pigments in floral parts and fruits which are important for pollination by insects and in seed dispersal. These pigments act as

attractants for pollinators and also as UV protectors. Flavones and flavonols can absorb UV light and thus protect plants from high exposure to UV. Flavonoids are also involved in plant-microbe interactions and male fertility (Han *et al*, 2006). Another function of flavonoids is as a defensive agent (phytollexis) against biotic and abiotic stress for example stilbenes. The interest in flavonoids has increased due to its potential health benefits for example isoflavonoids have been associated with anticancer benefits in foods such as soya beans and stilbenes in red wine is thought to contribute to reduced heart disease (Winkel-shirley, 2001). Phenylpropanoid metabolism also results in the production of compounds like lignin for strengthening of plant cell walls and vascular tissue as well as production of phenolics (signaling molecules).

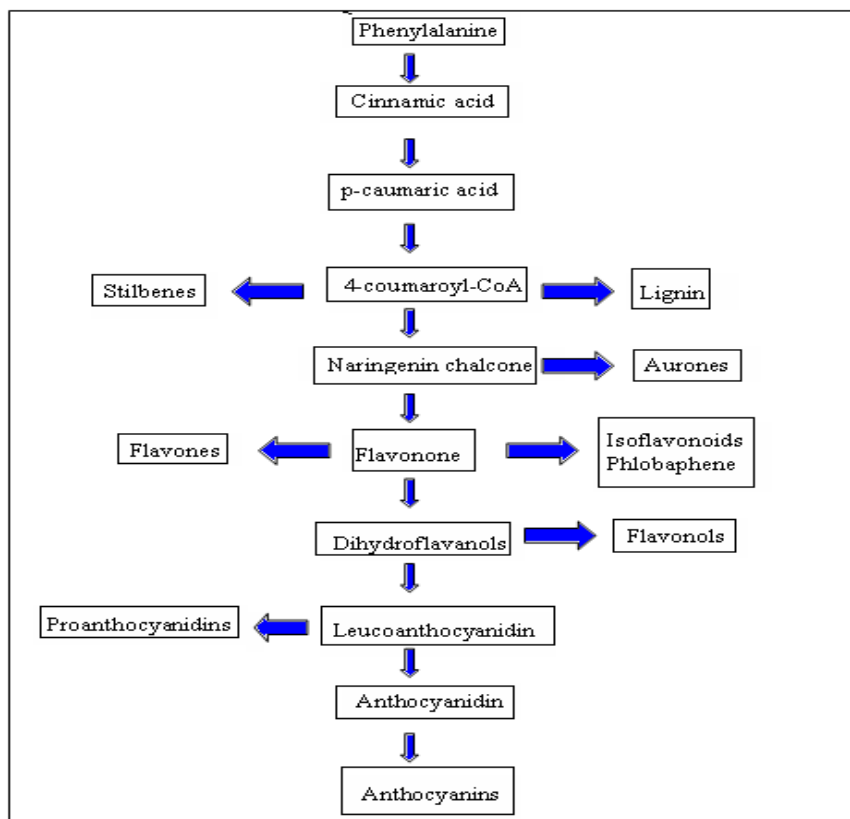


Figure 2.4: Phenylpropanoid pathway (Durbin *et al*, 2003)

2.4.1 Floral Anthocyanins in *Dendrobium* orchids

Different *Dendrobium* orchid species have been tested in order to identify what anthocyanins are present in their flowers. Three anthocyanins were identified in a variety of *Dendrobium* orchids; these were cyaniding, peonidin and pelargonidin. These anthocyanins were from flowers of various colours including pink, red, maroon, orange, bronze and brown. From this study the main anthocyanin common to all *Dendrobium* orchids was cyanidin and its derivative peonidin. It was also concluded that the flower colour in *Dendrobium* orchids was influenced by the level of anthocyanins present in the flowers (Kuehnle *et al*, 1997).

2.5 *R2R3-MYB* genes in *Dendrobium* orchid

The role of *MYB* genes in orchids was explored by (Wu *et al*, 2003), in a study that focused on the role of *MYB* genes in orchid flower development and anthocyanin biosynthesis. From this study on the orchid *Dendrobium* Woo Leng, 21 *MYB* genes were isolated and their expression was studied during orchid flower development. The results of the study showed that the *MYB9* gene was highly expressed in older flower buds but expressed at lower levels in younger flower buds and it was not expressed in the leaves or any other parts of the orchid plant. It was found that while the other *DwMYB* genes were expressed in all plant tissue, certain *DwMYB* genes including *DwMYB9* were tissue specific in their expression. *DwMYB9* was only expressed in mature flowers and buds, the inflorescence apex and was not expressed in the leaves.

This study was focused on specifically isolating regulatory *R2R3-MYB* genes involved in the anthocyanin biosynthetic pathway and in flower development. The conclusions made from the study were that *R2R3-MYB* genes comprise a large family in orchids. *DwMYB9*

was highly expressed in mature flowers but at lower levels in young flower buds and because the *DwMYB9* gene expression coincided with anthocyanin accumulation, it was possible that *DwMYB9* played a role in anthocyanin biosynthesis in the *Dendrobium* orchid Woo Leng. The function of the *DwMYB* genes remains largely unknown, thus the study provided an initial insight into the *MYB* gene family in orchids (Wu *et al*, 2003).

2.6 RNA interference (RNAi)

RNAi was first discovered in plants and later in *Caenorhabditis elegans* eventually leading to its discovery in several eukaryotic organisms. It is a naturally occurring biological process involving dsRNA to induce sequence specific gene silencing without affecting expression of other genes in the organism (Thackray, 2004). Small interfering RNAs (siRNAs) also play a crucial role in RNA silencing which can be referred to as post transcriptional gene silencing (PTGS) or cosuppression in plants, quelling in fungi and RNA interference in animals (Lu *et al*, 2004; Agarwal *et al*, 2003). There are clear differences in the RNAi process in plants and animals. For example it has been found in *Drosophila* embryos and mammalian cells that siRNAs produced are ~21 base pairs long however in plants and fungi two classes of lengths ~21bp and ~24bp have been found. The mechanism of RNAi is thought to be highly conserved in all eukaryotes including plants and animals. The siRNAs are produced through cleavage of dsRNA with an RNase III enzyme called dicer in animal cells or dicer-like (DCL) in plants. The siRNAs bind to a protein complex and are incorporated into an RNA-induced silencing complex (RISC) where siRNA plays a guiding role in sequence specific cleavage of target mRNA (Lu *et al*, 2004; Lehner *et al*, 2004). It has also been found that in certain organisms such as *C. elegans*, *Drosophila* and plants that the siRNA signal spreads along the mRNA target resulting in production of secondary siRNAs thus inducing transitive RNA silencing. This

could also be referred to as the organism specific systemic transmission of silencing from its site of initiation (Lu *et al*, 2004; Agarwal *et al*, 2003).

Experiments involving manipulation of gene expression in *Arabidopsis thaliana* and *C. elegans* revealed the process of RNAi and led to the discovery of the genes controlling the RNAi process. The RNAi process was thought to have evolved as a mechanism to eliminate foreign genes with high copy numbers that are present in cells, for example viral genes, transposable elements or plasmids, thus RNAi may have been used as a defense mechanism to silence expression of exogenously introduced genes or may be used directly or indirectly to regulate expression of endogenous genes (Lehner *et al*, 2004). As experiments were carried out in *C. elegans* it was found that injecting dsRNA into the worm resulted in degradation of the targeted mRNA. RNAi involves the specific degradation of endogenous RNA in the presence of homologous double stranded RNA (dsRNA) either locally injected or transcribed from an inverted repeat transgene. Injected RNA and transgenes expressing dsRNA trigger silencing of homologous genes in plants (Vaucheret *et al*, 2001). RNA plays a role in two types of homology dependent gene silencing (HDGS). They are posttranscriptional gene silencing (PTGS) which involves targeted degradation of homologous RNAs in the cytoplasm and RNA-directed DNA methylation (RdDM) which is induced by RNA derived from homologous DNA sequences at the genome level (Wei *et al*, 2001). Double stranded (dsRNA) plays a role in plant gene silencing by initiating both the RNA-degradation step of PTGS and RdDM. The dsRNA is processed by nuclease enzyme to produce small RNAs of 21-25 nucleotides. These are called small interfering RNAs or siRNAs (Ullu *et al*, 2002).

Experiments were done on *Petunia* to try to deepen the purple colour of flowers by introduction of transgenes. The results showed that many of the plants did not express the introduced transgene and instead if the transgene was homologous to the cellular gene then the endogenous copy was silenced. The term co-suppression was used to describe the process (Hammond *et al*, 2001).

2.6.1 Mechanism of RNAi

RNAi involves introduction of dsRNA into the plant cells where it is further processed. Once the dsRNA enters the cell, a nuclease enzyme called dicer digests the dsRNA and cleaves it into double stranded fragments of 21-25 base pairs referred to as small interfering RNA or siRNAs (Ullu *et al*, 2002). Dicer enzymes are essential for RNAi and recognize and process dsRNA. The siRNA are then recognized by a protein complex called RISC (RNA induced silencing complex) that recognizes and degrades target mRNA as shown in figure 2.5 (Milhavet *et al*, 2003; Vaucheret *et al*, 2001).

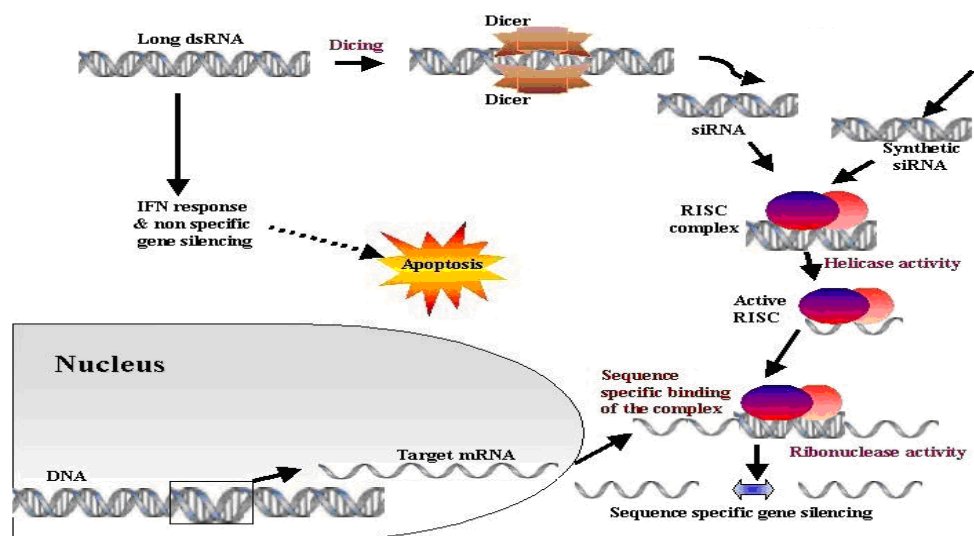


Figure 2.5: RNA interference mechanism (Srinivasan, 2005)

2.6.2 RNAi delivery methods in Plants

Several methods have been used to deliver dsRNA into cells to induce the RNAi pathway. Certain methods are more effective depending on the cell type in which RNAi is being induced. VIGS (virus induced gene silencing) is a method used in plants to induce RNAi and subsequently gene knockdown (Gould and Kramer, 2007). RNAi can also be achieved in plants through production of a hairpin RNA (hpRNA). Direct introduction of dsRNA or through a plasmid producing hpRNA has been shown to induce RNAi in plants. Vectors are designed with inverted repeats of the target gene spaced with an unrelated sequence and driven by a strong promoter to produce hpRNA (Kusaba, 2004). All these methods can be used to induce RNAi in plants; however their effectiveness varies in different plants.

2.6.2.1 Direct introduction of dsRNA produced in RNase deficient *E. coli*

Production of dsRNA in large quantities is very expensive and usually commercial kits are used. Based on *in vitro* transcription on linearised DNA templates or on PCR generated templates they can produce the required dsRNA. However to produce larger quantities of dsRNA that are less expensive, the *In-vivo* production of dsRNA in HT115 *E. coli* lacking ribonuclease III (RNase III) activity can be used instead (Ongvarrasoponea *et al*, 2007). It was found in the nematode worm *C. elegans* that when fed with HT115 *E. coli* strain lacking RNase III activity, high levels of specific dsRNA were produced that effectively induced a strong RNAi response (Tenllado *et al*, 2003). Thus the HT115 *E. coli* strain was used to produce large quantities of dsRNA e.g. viral-dsRNA was produced *in vivo* using this method to produce virus-derived dsRNA that could be used as a crude bacterial extract for specific gene silencing of virus infections in plants. The two viruses targeted in the study were Pepper mild mottle virus (PMMoV) and Plum pox virus (PPV). The *E. coli* strain HT115 can be induced to produce dsRNA through addition of isopropyl- β -D-

thiogalactopyranoside (IPTG) in the presence of a T7 promoter. Plasmid vectors containing T7, T3 or SP6 RNA polymerase promoters can be used to induce dsRNA production with IPTG (Ongvarrasoponea *et al*, 2007). Since the HT115 *E. coli* strain lacks RNase III enzyme, dsRNA can accumulate in bacterial cells without being degraded. When purified crude bacterial extracts were applied to virus infected plants, it was found that they were effective in causing degradation of viral RNA and providing protection against viral infections. The crude extracts that were sprayed on the plants surfaces also showed activity against viral RNA thus providing evidence that this technology can become widely applicable to protecting plants against viral infections (Tenllado *et al*, 2003).

2.6.2.2 Virus induced gene silencing in plants (VIGS)

Virus induced gene silencing (VIGS) is an RNA silencing techniques used in plants which involves use of viral vectors carrying a gene of interest to generate a dsRNA which induces silencing of a target gene. It was found that plants could overcome viral infections and even become resistant to later infections by related viruses. These observations gave the first indication that PTGS was involved in antiviral defense mechanisms in plants (Burch-Smith *et al*, 2006). When the virus replicates within the plant, double stranded chimeric intermediates are produced. The plant recognizes these as foreign and cuts the dsRNA to form siRNA which forms an RNA-induced silencing complex that degrades other sequences that have identical homologous thereby leading to gene silencing (Chen *et al*, 2005).

VIGS vectors are produced by modifying viral genomes. Usually RNA viruses that infect several plant species are used; however DNA viruses have been used as well. The most widely used VIGS vector is from the tobacco rattle virus (*TRV*). The advantages of using

this *TRV* in VIGS is that it can infect a wide range of plants and the virus can infect the meristem of the host therefore it can be used to study flowering and fruit development (Burch-Smith *et al*, 2006). Introduction of the VIGS vector into the plant is through *Agrobacterium*-mediated transformation where the vector is placed between the T-DNA borders. DNA viruses used for VIGS are derived from the bipartite cabbage leaf curl geminivirus (CbLCV). This vector has limited use perhaps due to difficulty with introducing it into the plant through particle bombardment and since it can only accommodate a limited insert size of 800bp in the CbLCV vector. The VIGS technique was applied to the plant *Aquilegia vulgaris* using vector from the tobacco rattle virus (*TRV*). Silencing resulted in reduction of photoprotective carotenoid proteins and breakdown of chlorophyll pigments and also inhibiting development of the purple wild type flower colour. These results showed that the VIGS method is useful for studying gene function and also in gene silencing (Gould & Kramer, 2007).

CHAPTER 3: METHODS

3.0 Sample collection

Orchid plants (*Dendrobium* Sonia) were purchased from a commercial orchid nursery at the flowering stage or with inflorescence buds present. The orchids were kept in a growth room with controlled 12 hour photoperiod. They were watered daily. Individual inflorescences were collected within the first or second day of opening and ground in liquid nitrogen to extract total RNA.

3.1 RNA extraction

Total RNA was extracted from orchid flowers using the Qiagen plant mini kit (Qiagen, USA). Approximately 100mg of flowers tissue was wrapped in aluminum foil and placed in liquid nitrogen. Then it was ground using a motor and pestle. This was then added to 450ul of buffer RLT that contained guanidine thiocyanate and 4.5ul of β -mercapthoethanol. The lysate was vortexed vigorously and then transferred to a lilac spin column. This was centrifuged for 2 minutes at 10,000 x g. The supernatant of the flow through was then transferred to a new 2ml tube without disturbing the pellet in the collection tube. A 0.5 volume of ethanol was added to the lysate and mixed by pipetting. This mixture was transferred to a pink spin column in a 2ml collection tube for 15 seconds at 10,000 x g. The flow through was discarded. 700ul of buffer RW1 was added to the spin column and centrifuged for 15 seconds at 10,000 x g. The flow through was discarded. Buffer RPE (500ul) was added to the spin column and centrifuged for 15 seconds at 10,000 x g. The flow through was discarded. Another 500ul of buffer RPE was added and centrifuged for 2 minutes at 10,000 x g. The flow through was discarded. The spin column was centrifuged for 1 minute at 10,000 x g and the collection tube discarded. The spin

column was placed in a new 1.5ml tube and 50ul of RNase free water was added directly to the spin column. This was centrifuged for 1 minute at 10,000 x g to elute the RNA.

3.2 Agarose Gel Electrophoresis

A 1% agarose gel was made by mixing 0.3g of agarose powder in 30ml of 1X TBE buffer. This was placed in the microwave for 1 minute to melt the agarose powder and then 1ul (~1ug/ml) of ethidium bromide was added. The gel was poured into a mould and allowed to set for 30 minutes. It was then placed in the electrophoresis chamber and immersed in TBE buffer. The RNA samples were loaded into the gel and it was run for 25 minutes at 120 volts.

3.3 Reverse Transcriptase PCR (RT-PCR)

RT-PCR was carried out on the RNA using the following primers,

MYB9 Forward primer: 5' CCA ACC ATG GAG AGG GAG TA 3'

MYB9 Reverse primer: 5' GGA CCA AGA AAT CCT CCA CA 3'

The *DwMYB9* gene sequence with accession number AF485900 (Wu *et al*, 2003) was obtained from the NCBI website ([http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=Nucleotide &val=28628962](http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=Nucleotide&val=28628962)). This sequence was used to design *MYB9* primers through the primer3 tool (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_results.cgi).

The Access RT-PCR kit (Promega, USA) was used. The reaction conditions were as follows.

TABLE 3.1
The reaction volumes used for RT-PCR

For a 50ul Reaction Volume		For a 25ul Reaction Volume (Scale Down of the reaction)	
AMV/ <i>Tfl</i> 5X Reaction Buffer	10ul	AMV/ <i>Tfl</i> 5X Reaction Buffer	5ul
25mM MgSO ₄	2ul	25mM MgSO ₄	1ul
dNTP Mix(10mM each)	1ul	dNTP Mix(10mM each)	0.5ul
Forward Primer (100uM)	2.4ul	Forward Primer (100uM)	1.2ul
Reverse Primer (100uM)	2.4ul	Reverse Primer (100uM)	1.2ul
AMV Reverse Transcriptase (5u/ul)	1ul	AMV Reverse Transcriptase (5u/ul)	0.5ul
<i>Tfl</i> DNA Polymerase(5u/ul)	1ul	<i>Tfl</i> DNA Polymerase(5u/ul)	0.5ul
RNA Sample or Control (ug)	2.6ul	RNA Sample or Control (ug)	1.3ul
Nuclease-Free Water	27.6ul	Nuclease-Free Water	13.8ul
Final Volume	50ul	Final Volume	25ul

TABLE 3.2
The reaction volumes used for positive and negative controls on RT-PCR reaction

Positive Control(use control primers and control RNA)		Negative Control(use nuclease free water instead of RNA)	
AMV/ <i>Tfl</i> 5X Reaction Buffer	5ul	AMV/ <i>Tfl</i> 5X Reaction Buffer	5ul
25mM MgSO ₄	2ul	25mM MgSO ₄	2ul
dNTP Mix(10mM each)	0.5ul	dNTP Mix(10mM each)	0.5ul
Downstream Control Primer(50pmol)	1.2ul	Downstream Control Primer (50pmol)	1.2ul
Upstream Control Primer (50pmol)	1.2ul	Upstream Control Primer (50pmol)	1.2ul
AMV Reverse Transcriptase (5u/ul)	0.5ul	AMV Reverse Transcriptase (5u/ul)	0.5ul
<i>Tfl</i> DNA Polymerase (5u/ul)	0.5ul	<i>Tfl</i> DNA Polymerase (5u/ul)	0.5ul
RNA Control (ug)	1.3ul	RNA (substitute with Water)	1.3ul
Nuclease-Free Water	13.8ul	Nuclease-Free Water	13.8ul
Final Volume	25ul	Final Volume	25ul

* The positive control for the RT-PCR reaction mixture uses control primers and control RNA and should give an amplimer of 323bp or occasionally 220bp.

* The negative control for the RT-PCR reaction mixture uses nuclease free water instead of RNA while all other reagents are the same as positive control, no bands should be observed for this reaction.

Actin positive control (Shows bands in gel if RNA is present in the sample)

ACTIN Forward primer: 5' GGA ATT CCT CCA GCT GCC ACT TAC TCC 3'

ACTIN Reverse primer: 5' AGA GCT CTT GAG CAG GGT AGC ACT CTT GG 3'

Reaction without Reverse Transcriptase

If there is DNA contamination in the RNA sample then it will show as a band on the gel therefore to rule out DNA amplification and ensure that only the RNA is being amplified, reverse transcriptase enzyme is not added to the mixture.

3.4 Agarose Gel DNA Extraction System

The MEGAquick-spin Agarose gel extraction system from (Intronbio) was used to extract the 500bp DNA fragment from the gel. The sample was first loaded into the gel and run for 25 minutes. It was excised from the gel through visualization under a UV lamp and placed in a 1.5ml centrifuge tube that was weighed. The weight of the gel was determined and the amount of BNL buffer to be added was calculated (300ul per 100mg of agarose gel slice). The mixture was vortexed and incubated at 55°C for 10 minutes to dissolve the gel slice completely. The dissolved gel mixture was transferred to a spin column and collection tube, where it was centrifuged for 1 minute at 10,000 x g. The flow through was discarded and 700ul of washing buffer was added to the spin column. It was centrifuged for 1 minute at 10,000 x g after which the flow through was discarded and then centrifuged for another 1 minute. The spin column was transferred into a new 1.5ml collection tube and 30-100ul of elution buffer was added. This was incubated at room temperature for 1 minute and then centrifuged for 1 minute at 10,000 x g. The spin column was discarded and the eluted DNA was then used for cloning.

3.5 Cloning of PCR products

Reactions were performed using the TOPO TA KIT (Invitrogen, USA) following the manufacturers protocol.

3.5.1 Cloning Reaction

TOPO vector (10ng/ul)	1ul
NaCl (1.2 M) & MgCl ₂ (0.06 M)	1ul
Water	2ul
PCR product	2ul (~ 1000ng/ul)
Total	6ul

All the above reagents were mixed and left to incubate for 5 minutes.

3.5.2 Transformation of *E. coli*

E. coli TOP10 competent cells (TOPO TA KIT (Invitrogen, USA)) were thawed and 2ul of the reaction mix was added. This was mixed gently and left to incubate on ice for 30 minutes. The cells were then placed in a 42⁰C water bath for 30 seconds to heat shock them. Then 250ul of S.O.C medium was added to the cells and this was incubated in a water bath at 37⁰C and 225rpm for 1 hour. About 10-50ul was spread on warmed LB agar plates containing X-gal and 50ug/ml ampicillin, incubated overnight at 37⁰C and white colonies were picked for analysis.

3.6 Screening of clones (Colony PCR)

To screen the transformed bacterial colonies and see which ones contain the correct size insert (500bp PCR product), colony PCR was performed. A bacterial suspension was made in 10ul of distilled water for each colony. This was heated in a water bath at 94⁰C for 10 minutes to lyse the bacterial cells and release the plasmid DNA. A PCR reaction was then performed on the Plasmid DNA using the M13 forward and reverse primers. This resulted

in amplification of the region with the insert. From here only colonies with the approximate length corresponding to that of the insert were selected for plasmid extraction.

Use M13 primers provided and see if band length is close to approx. 700bp

M13 Forward primer (-20): 5'-GTAAAACGACGGCCAG-3'

M13 Reverse primer: 5'-CAGGAAACAGCTATGAC-3'

3.7 Plasmid Extraction Kit

Plasmid DNA extractions were performed using a commercial kit (Invitrogen USA). About 1-5ml of an overnight culture in LB broth was pelleted by centrifuging at 4000rpm for 4 minutes. All the media was removed from the cell pellet and it was resuspended in 250ul resuspension buffer (R3) with RNase A. Lysis buffer (250ul) was added to cells and mixed gently by inverting the tube 5 times. The tube was incubated for 5 minutes at room temperature and 350ul of precipitation buffer (N4) was added. This was mixed immediately until the solution was homogenous and centrifuged at 9600 x g for 10 minutes at room temperature. The supernatant was loaded onto a spin column and placed in a 2ml wash tube. It was centrifuged at 9600 x g for 1 minute. Flow through was discarded and the spin column was placed back in the wash tube. Wash buffer (500ul) (W10) was added with ethanol to the column. It was incubated for 1 minute at room temperature, centrifuged at 9600 x g for 1 minute and flow through was discarded. Wash buffer (700ul) (W9) with ethanol was added to the column. This was centrifuged at 9600 x g for 1 minute and the flow through discarded. The spin column was placed in a clean 1.5ml recovery tube and 75ul of preheated TE buffer was added to the center of the column and incubated for 1 minute at room temperature. This was centrifuged at 9600 x g for 2 minutes and the column was discarded. The plasmid DNA was collected in the recovery tube.

3.8 DNA Sequencing and Analysis

The DNA sequence of the inserts from extracted plasmid samples was determined using a commercial sequencing service. DNA sequences were analyzed using the BioEdit software suite (www.mbio.ncsu.edu/BioEdit/BioEdit.zip). The sequences were converted into FASTA format and the basic alignment tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to identify the sequences based on similarity to the *MYB9* gene from *Dendrobium* species.

3.9 Restriction enzyme digestion

Reaction conditions

10X Buffer	2ul
Enzymes	0.1ul (2units)
Plasmid	1ul (~ 1ug/ul)
dH ₂ O	16.9ul
Total	20ul

Nco1= 10000units/ml
NEB buffer 3

The plasmid samples were subjected to restriction enzyme digestion by incubating the reaction mixture at 37⁰C for 1 hour. Then the samples were incubated at 65⁰C for 20 minutes to inactivate the enzyme.

3.10 Preparation of *E. coli* HT115 Competent cells

The plasmids containing the *MYB9* forward and reverse inserts were transformed into HT115 *E. coli*. This bacterial strain can transcribe ssRNA from the T7 promoter region in the plasmid and also the insert. The HT115 cells were first made competent by addition of calcium chloride (CaCl₂). The stock of HT115 was taken from -80⁰C freezer and streaked onto LB agar with 20ug/ml tetracycline. This was left to grow overnight in a 37⁰C

incubator. A single colony was then selected and grown in 3-5ml LB broth with 20ug/ml tetracycline overnight at 37⁰C in a water bath at 220rpm. Then 25ml of LB broth with 20ug/ml tetracycline was inoculated with 250ul of overnight culture to make a 1:100 dilution. This was again incubated at 37⁰C with shaking at 220rpm until exponential phase was reached, about OD₅₉₅=0.4. The culture was centrifuged at 2400 x g for 10 minutes at 4⁰C. The pellet was resuspended in 12.5ml ice cold 50mM CaCl₂. This was left on ice for 30 minutes and then centrifuged for 10 minutes at 2400 x g at 4⁰C. The pellet was resuspended in 2.5ml CaCl₂ and 200ul aliquots were made. Glycerol was added and the cells stored in -80⁰C freezer.

3.11 Transformation of *E. coli* HT115 competent cells

The HT115 competent cells (200ul) were thawed on ice and 5ul of plasmid was added to each tube. This was mixed gently and left on ice for 30 minutes. Heat shock was done in a 37⁰C water bath for 1 minute and then the tube placed on ice for another 2 minutes. LB broth with 100ug/ml ampicillin and 12.5ug/ml tetracycline was then added (800ul) and incubated in a 37⁰C water bath at 220rpm. Then 500ul was plated onto LB agar with 100ug/ml ampicillin and 12.5ug/ml tetracycline. This was placed in a 37⁰C incubator overnight.

3.12 Induction of bacterial RNA expression

Single colonies of HT115 containing the plasmid harboring the constructs were inoculated into 3-5ml of LB broth with 100ug/ml ampicillin and 12.5ug/ml tetracycline. This was grown overnight at 37⁰C in a water bath at 220rpm. The culture was diluted 100 fold with 2xYT broth with 100ug/ml ampicillin and 12.5ug/ml tetracycline. This was allowed to grow till OD₅₉₅= 0.5 (3-4hrs). The HT115 T7 polymerase gene was then induced to

transcribe ssRNA by addition of 0.4mM IPTG (10ul). The culture was incubated with shaking for 2-4hrs at 37⁰C. The culture was spiked with additional antibiotics (another 100ug/ml ampicillin, 12.5ug/ml tetracycline and 0.4mM IPTG). This was again incubated with shaking for another 20 minutes. Alkaline lysis was then performed.

3.13 Alkaline lysis of bacterial cells

The IPTG induced HT115 cells (25ml) were pelleted by centrifuging at 3200 x g for 6 minutes at 4⁰C and resuspended in 9.4ml of resuspension buffer (10mM EDTA, 50mM Tris Base to pH 8 with HCL). Then lysis buffer (0.2M NaOH and 1% SDS) was added to the mixture. The cells were pelleted at 3200 x g for 6 minutes at 4⁰C. The supernatant was removed and stored at -80⁰C.

3.14 Annealing of ssRNA

The concentration of ssRNA in the crude bacterial extract was determined using a spectrophotometer ((A₂₆₀/A₂₈₀) nm wavelength) and the samples diluted accordingly so that equal concentrations of both forward and reverse ssRNA could be mixed together. 25mM of sodium phosphate was added and the mixture heated up to 95⁰C for 3mins. This was allowed to cool to 37⁰C for 30 minutes in order for annealing to occur.

3.15 Functional studies on orchid buds (RNAi)

The crude bacterial extracts were tested on orchid buds. The orchid plants were divided into 5 treatment groups. The treatments were as follows,

- 1) Crude bacterial extract from sample 1S (ssRNA reverse orientation)
- 2) Crude bacterial extract from sample 2B (ssRNA forward orientation)
- 3) Samples 1S and 2B annealed to form dsRNA

- 4) Untreated control (no RNA treatment)
- 5) Negative control (HT115 bacterial extract without an insert)

Each treatment group contained 6 orchid plants that were classified based on the size of buds in each inflorescence. Plant number 1 in each group had buds that were more mature and larger in size while plant number 6 in each group had buds that were less mature and smaller. Thus from plant 1-6 the size of buds was decreasing. The first treatment on orchid buds involved spreading the bacterial extract (~0.8-1ug/ul of each extract) onto the surface the buds with cellite that was mixed into the bacterial extract to act as an abrasive that would slightly damage the outer cells on the orchid buds thus allowing the RNA to enter the cells. The crude bacterial extract was left on the buds for approximately 5 minutes after which it was washed off with distilled water. The orchids were watered everyday and allowed to flower.

3.16 Analysis of treatments on orchid buds using semi quantitative RT-PCR

Orchid buds from the same treatment group that flowered on the same day or at least within one day of each other were pooled together and the total RNA was extracted from the flowers. In the case of *Dendrobium* Sonia flowers, RNA from sepals, petals and lips of each flower was extracted separately. A minimum of three flower samples from each treatment group were pooled together and this was repeated three times for each group of samples. The absorbance readings ((A_{260}/A_{280}) nm wavelength) were taken for total RNA extracted from flower samples to determine the concentration. Then the RNA samples were diluted accordingly to equal concentrations and RT-PCR was performed using the *MYB9* and actin primers. Actin was used as an internal standard for each RNA sample. The PCR product was analysed on a 1% agarose gel and the intensity of the bands for each sample was compared. The area of a band is the number of pixels in the region and the

sum is the total value of all pixel gray levels in a region therefore it can be interpreted as the total signal (intensity) from a region representing the total DNA in each band. The average is the sum divided by the area and this represents the average signal level from a region or the average DNA signal in a region. Therefore the average gives the intensity value for the band of interest. The band intensity was measured using ImageJ band densitometry analysis software (<http://rsb.info.nih.gov/nih-image/>). The data obtained was normalized to the actin standard and the relative intensity values were transferred to Microsoft excel where graphs were made to represent the data. The RNA secondary structure of *MYB9* ssRNA was determined using the Mfold software (<http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi>).

CHAPTER 4: RESULTS

4.0 Identification of *MYB9* gene in *Dendrobium* Sonia orchids

The picture below (figure 4.1) shows the total RNA extracted from *Dendrobium* Sonia orchid flowers. There are two distinct RNA bands, the upper band indicating 20S rRNA and the lower band 18S rRNA. Usually intact total RNA will show these two bands.

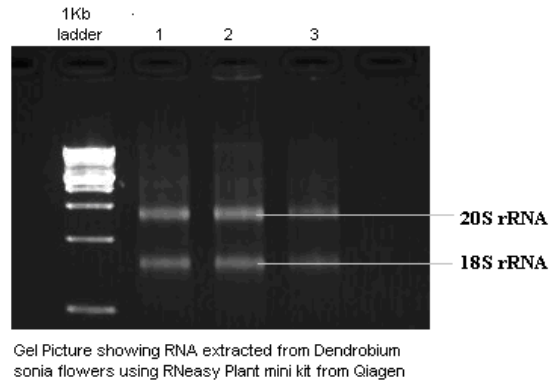


Figure 4.1: RNA bands on a 1% Agarose gel extracted from *Dendrobium* Sonia flowers

Lane 1, 2 & 3- RNA extracted from *Dendrobium* Sonia flowers

To isolate the *MYB9* gene from orchid flowers, a one step RT-PCR was performed on RNA extracted from flowers. A 500bp product was amplified using *MYB9* primers designed from the *MYB9* sequences of *Dendrobium* Woo Leng (Genbank accession number AF485900). DNA contamination was ruled out as no bands were observed when reverse transcriptase was left out of the reaction. Actin was used as a positive control to confirm presence of RNA in the sample. Several bands were observed during RT-PCR however the 500bp band was most prominent (Figure 4.2). The gradient did not show much difference in the bands and the temperature ranged from 57⁰C to 65⁰C, indicating that the *MYB9* primer had a wide range of temperatures for annealing to the region of interest.

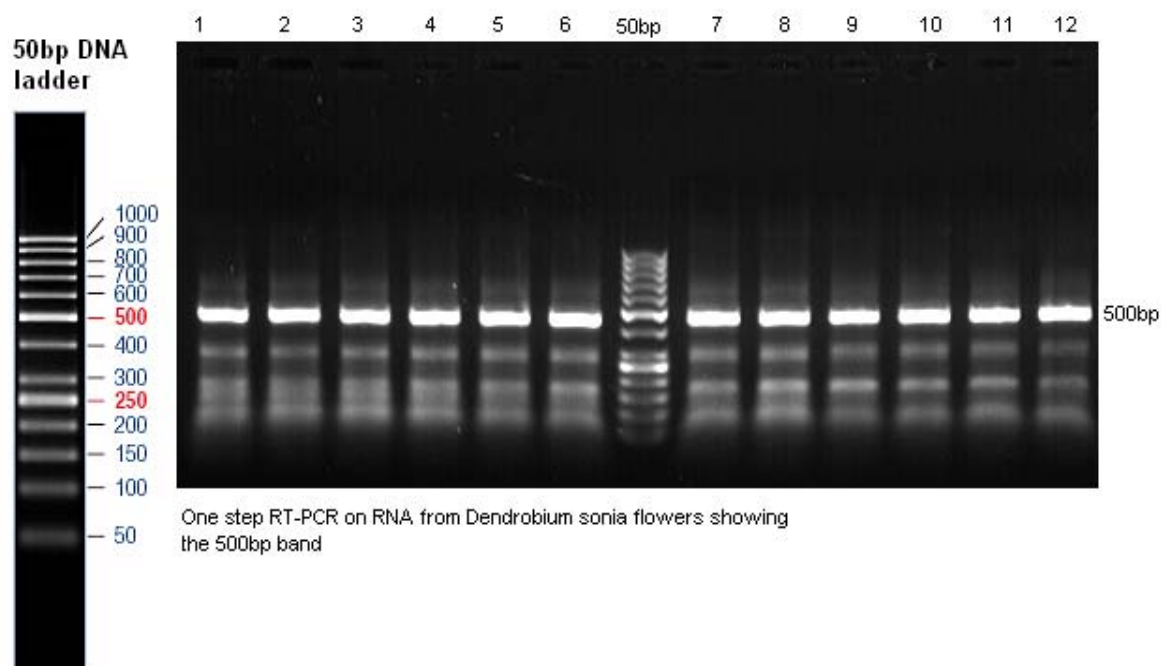


Figure 4.2: RT-PCR gradient using *MYB9* primers on RNA from *Dendrobium Sonia* flowers

Lanes 1-12- RNA sample containing 500bp band
 50bp- 50 base pair DNA ladder

The 500bp band (figure 4.2) was excised from the gel and purified using the MEGAquick-spin Agarose gel extraction system (Intronbio). This purified RT-PCR product was used in cloning and the white colonies (those containing insert) were screened by colony PCR (figure 4.3 and 4.4) to select only those that would have an insert of the required size of approximately 700bp (to include the whole region between the M13 forward and reverse primers). Around 30-40 colonies were obtained after each round of cloning of which approximately 10-20 white colonies were present. Altogether around 40-50 white colonies were screened by colony PCR. Clones with inserts of 700bp (about 27 colonies) were chosen for plasmid extraction and DNA sequencing. Colonies containing the *MYB9* gene

were subjected to restriction enzyme digestion to determine the orientation of the *MYB9* gene within the plasmid.

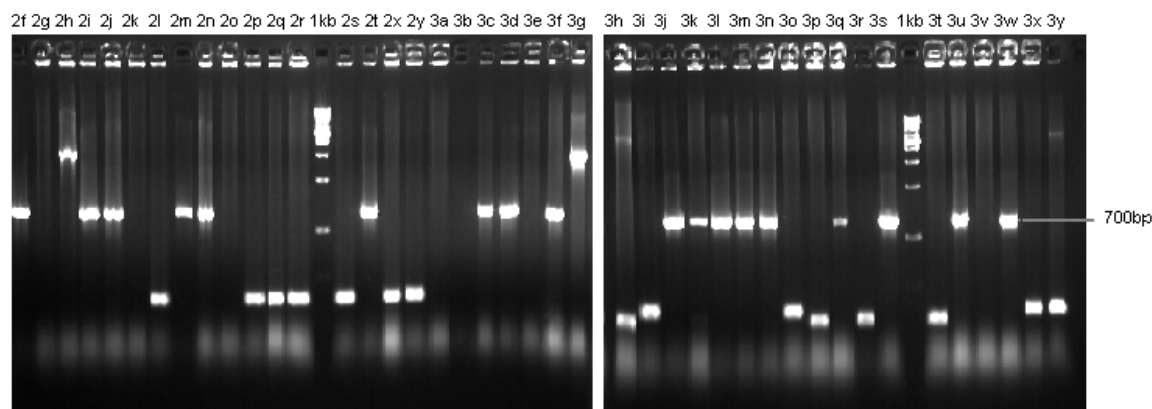


Figure 4.3: Colony PCR on clones transformed with purified PCR product showing plasmid containing the insert as 700bp bands

Lanes 2f, 2i, 2j, 2m, 2n, 2t, 3c, 3d, 3f, 3j, 3k, 3l, 3m, 3n, 3q, 3s, 3u, 3w- plasmid samples containing insert and showing 700bp bands

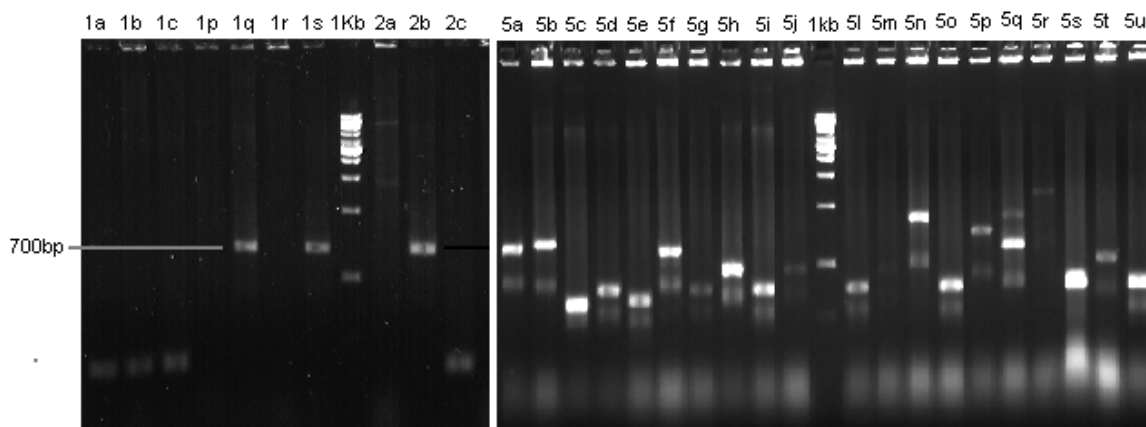


Figure 4.4: Colony PCR on clones transformed with fresh PCR product showing bands of different sizes

Lanes 1q, 1s, 2b, 5a, 5b, 5f, 5q- plasmid samples containing insert and showing 700bp bands

The sequencing results were converted to FASTA format and analyzed by BLAST to find similarity to other known nucleotide sequences in the database. The 500bp insert from sample 2B had a 98% similarity to the *MYB9* gene in *Dendrobium* species as shown in figure 4.5. Other plasmids showed similar results confirming that a *MYB9* sequence was isolated from the flowers.

Query ID lc|3684
Description 2B_Jen_M13R
Molecule type nucleic acid
Query Length 936 Database Name nr
Description All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences) See details
Program BLASTN 2.2.18+ Citation

>gil28628962[gb|AF485900.1| Dendrobium sp. XMWV-2002-9 MYB9 (MYB9) mRNA, complete cds
Length=649

Score = 626 bits (316), Expect = 3e-176
Identities = 334/340 (98%), Gaps = 0/340 (0%)
Strand=Plus/Minus

Query 90 TCTTCAATGCTACTTGTATGGCTTGTGCAGGCCTCATCAATCATCATTGGCTCGAGTAA 149
|||||
Sbjct 394 TCTTCAATGCTACTTGTATGGCTTGTGCAGGCCTCATCAATGATCATTGGCTCGAGTAA 335

Query 150 TCGACTGCATCGCCATTCTTAACCTTCTTCTGGATTCTAGTCTCCAGTAGTTCTTGATC 209
|||||
Sbjct 334 TCGACTGCATCGCCATTCTTAACCTTCTTTGAATTCTAGTCTCCAGTAGTTCTTGATC 275

Query 210 TCATTATCAGTCCTTCTGGAAGTTGCCTCGCAATTTTGACCACTGTTTCCCCACCTG 269
|||||
Sbjct 274 TCATTATCAGTCCTTCTGGAAGTTGCCTCGCAATTTTGACCACTGTTTCCCCACCTG 215

Query 270 GCATGCAACTCCATGATCAACACCTGCTCCTCCGGCGTTATGTTCCCCCTCCTCAGTCT 329
|||||
Sbjct 214 GCATGCAACTCCATGATCAGCAGCTGCTCCTCCGGTGTTATGTTCCCCCTCCTCAGTCT 155

Query 330 GGCCTCAGATAGTTTAGCCATCGAAGCCGGCAGCTCTTCCCGTCCTCTTCAAACCTGCA 389
|||||
Sbjct 154 GGCCTCAGATAGTTTAGCCATCGAAGCCGGCAGCTCTTCCCGTCCTCTTCAAACCTGCA 95

Query 390 GATTGGCAAGATTATTCCATACTCCCTCTCCATGGTTGG 429
|||||
Sbjct 94 GATTGGCAAGATTATTCCATACTCCCTCTCCATGGTTGG 55

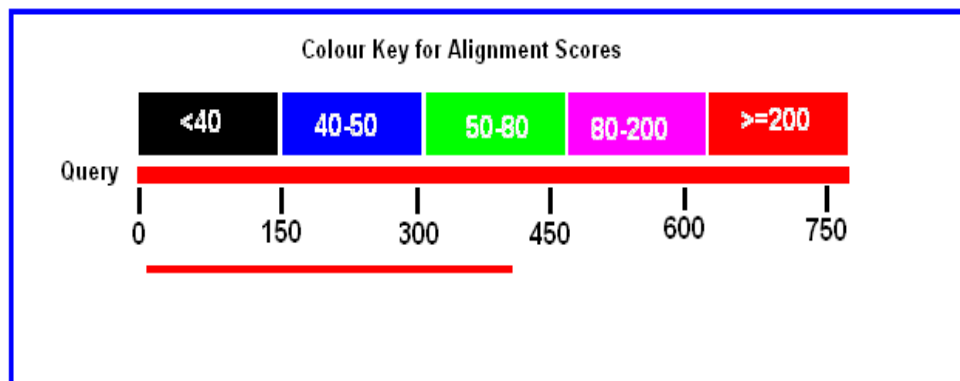


Figure 4.5: Results of BLAST on sample 2B showing a 98% similarity to the *Dendrobium MYB9* sequence

4.1 Determination of the orientation of *MYB9* gene within plasmids

Restriction enzyme digestion of the plasmid was used to determine the orientation of the *MYB9* gene within the pCR2.1- TOPO plasmid. The BioEdit program was used to find restriction enzyme sites on the *MYB9* insert. A restriction enzyme table listing all restriction sites for sample 2B and map of the restriction enzyme sites generated in BioEdit are shown in appendix C. The enzyme *NcoI* was chosen because it had one restriction site within the insert and another within the plasmid thus would produce two fragments of different lengths for each of the possible orientations of insert, as shown in the plasmids maps in Figure 4.6. From figure 4.6, it was calculated that the two fragments produced through restriction enzyme digestion with *NcoI* would either have sizes of 1665bp and 2766bp or 2038bp and 2393bp. These predictions were confirmed as shown in figure 4.7 by restriction enzyme digestion on plasmid samples. The samples with the *MYB9* insert in forward or reverse orientation gave the approximate size fragments that were calculated.

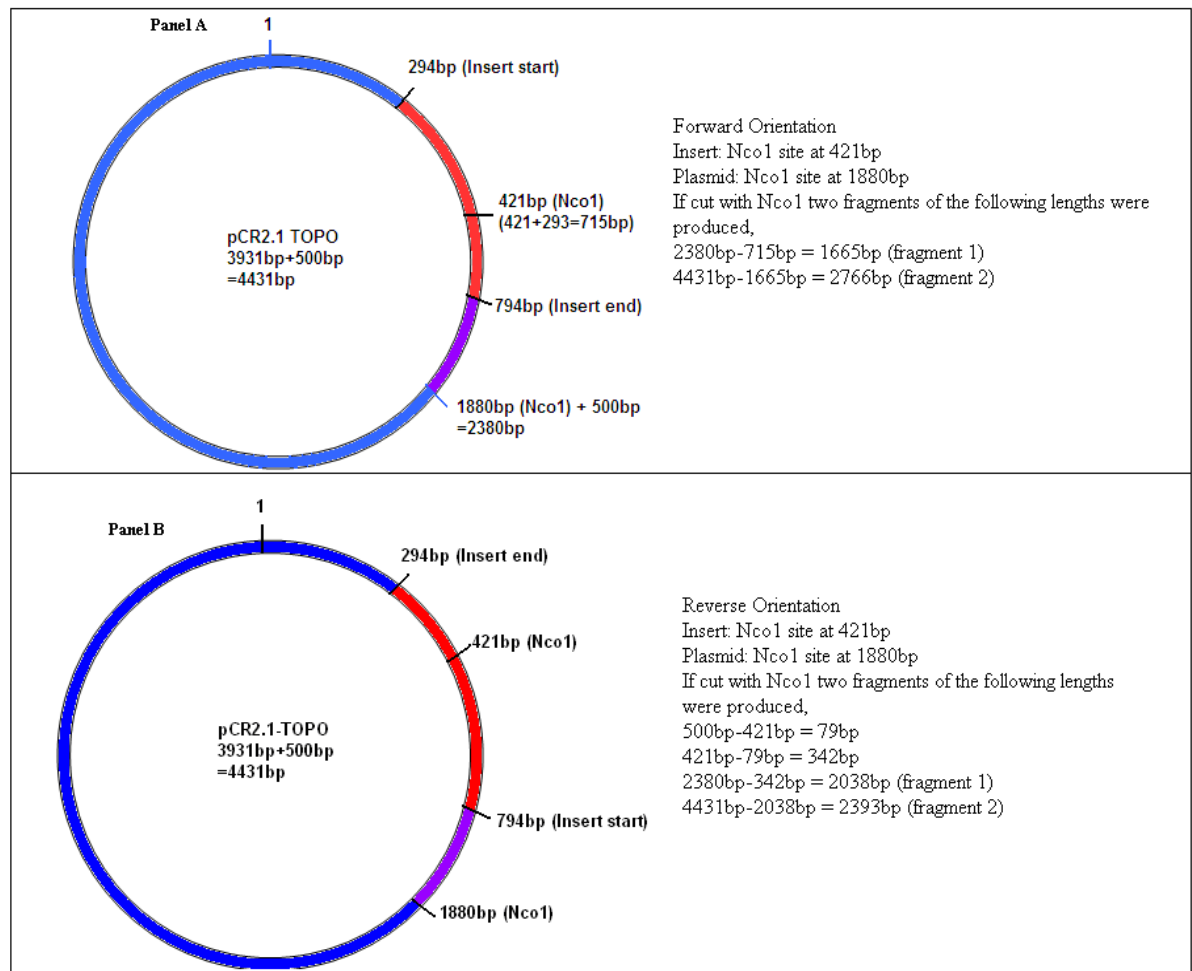
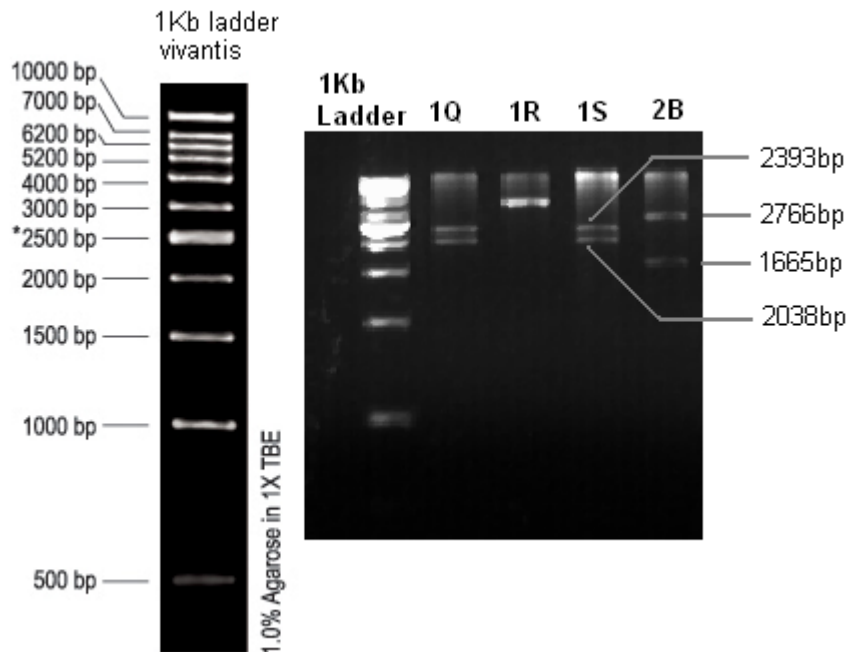


Figure 4.6: Restriction enzyme digestion with *NcoI* to detect the orientation of *MYB9* gene: Panel A and B show the two possible orientations of the *MYB9* gene inserts with the different restriction patterns for *NcoI* digest for each. Panel A “forward orientation” would result in two fragments of sizes 1665bp and 2766bp and Panel B “reverse orientation” would result in two fragments of sizes 2038bp and 2393bp



(www.vivantis.com)

Figure 4.7: Agarose gel electrophoresis of plasmid samples digested with *NcoI*

Lanes 1Q, 1S- show band sizes 2393bp and 2038bp

Lane 1R- shows band size 4431bp

Lane 2B- show band sizes 2766bp and 1665bp

These results of the *NcoI* digestion (figure 4.7) were used to determine which plasmid samples contain the *MYB9* gene in forward or reverse orientations and these were sent for sequencing to confirm the results. As shown in figure 4.7, samples 1Q and 1S produced fragments with the approximate lengths of 2393bp and 2038bp which correspond to the expected fragment lengths in figure 4.6, indicating that the *MYB9* gene was in the reverse orientation in these samples. Sample 2B had fragments with approximate lengths of 2766bp and 1665bp (figure 4.7), indicating that the *MYB9* gene was in the forward orientation. Two plasmid samples 1S and 2B were selected for induction of bacterial RNA expression representing the *MYB9* gene sequences in reverse and forward orientation respectively.

4.2 RNA interference studies carried out on orchid buds

Each treatment group contained 6 orchid plants that were treated with the following crude bacterial extract containing RNA. Each treatment used on orchids had an RNA concentration of about 0.8-1ug/ul.

- 1) Treated with sample 1Sb: $OD_{260/280} = 1.391\text{ug/ul}$. This was the reverse orientation of the *MYB9* gene.
- 2) Treated with sample 2Ba: $OD_{260/280} = 1.520\text{ug/ul}$. This was the forward orientation of the *MYB9* gene
- 3) Treated with sample dsRNAa: $OD_{260/280} = 0.782\text{ug/ul}$. This consisted of samples 1Sd and 2Bd that were annealed together to form dsRNAa. (1Sd: $OD_{260/280} = 0.819\text{ug/ul}$) (2Bb: $OD_{260/280} = 0.948\text{ug/ul}$)
- 4) Untreated control for each bud stage 1-6 (no RNA treatment)
- 5) Treated with the negative control (HT115 bacterial extract without *MYB9* insert ($OD_{260/280} = 0.865\text{ug/ul}$)).

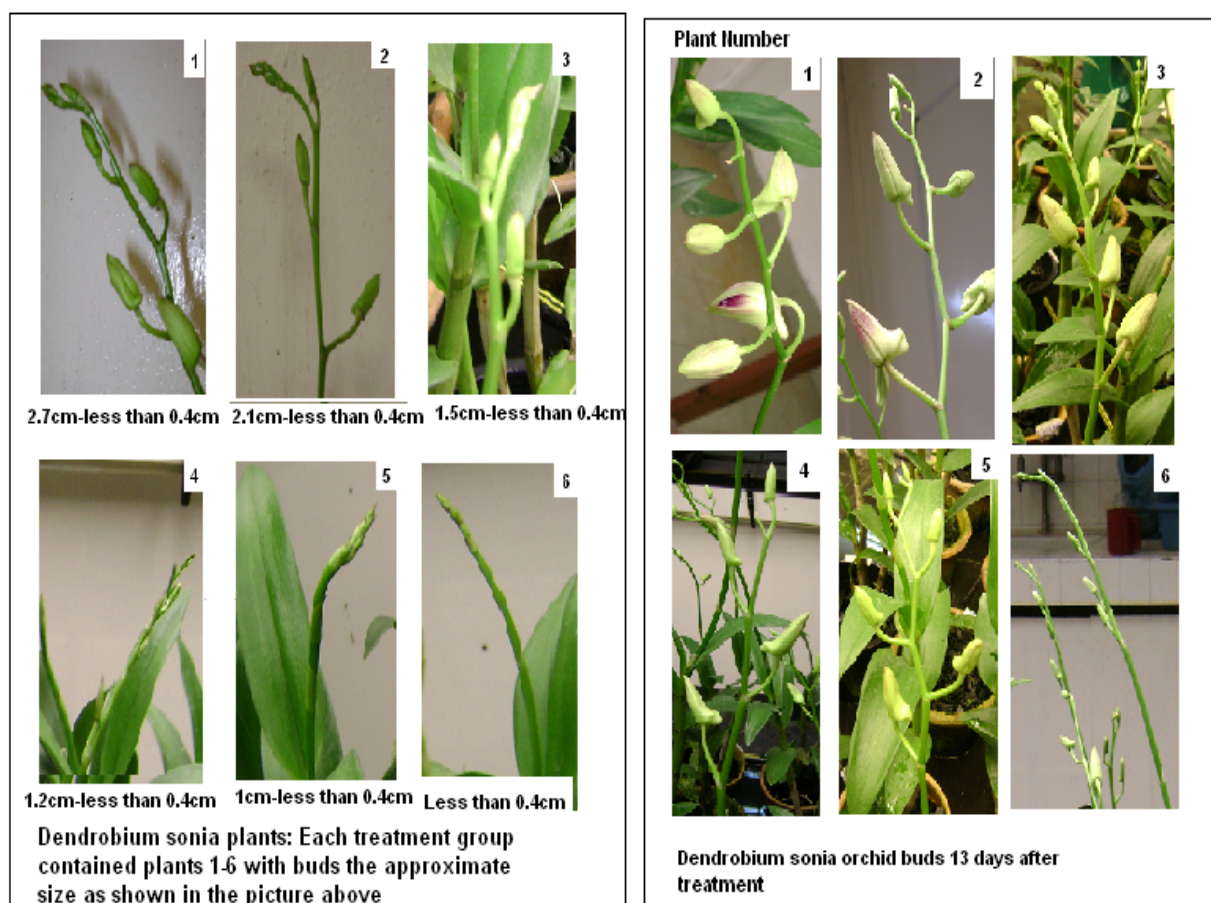


Figure 4.8: The sizes of buds from orchid plants in each treatment group

Each treatment group 1-6 contained orchid plants with buds of different sizes as shown in figure 4.8. The reason for selecting different sized buds was to observe if the size of the buds when treated affected the expression of the *MYB9* gene in the orchids. When the buds of the orchids flowered, they were observed for any colour changes to determine whether the crude bacterial extract had any effect on colour production in the *Dendrobium Sonia* orchid (Figure 4.9). However there were no observations of significant colour changes in any of the flowers. It was also observed that several buds died before flowering.

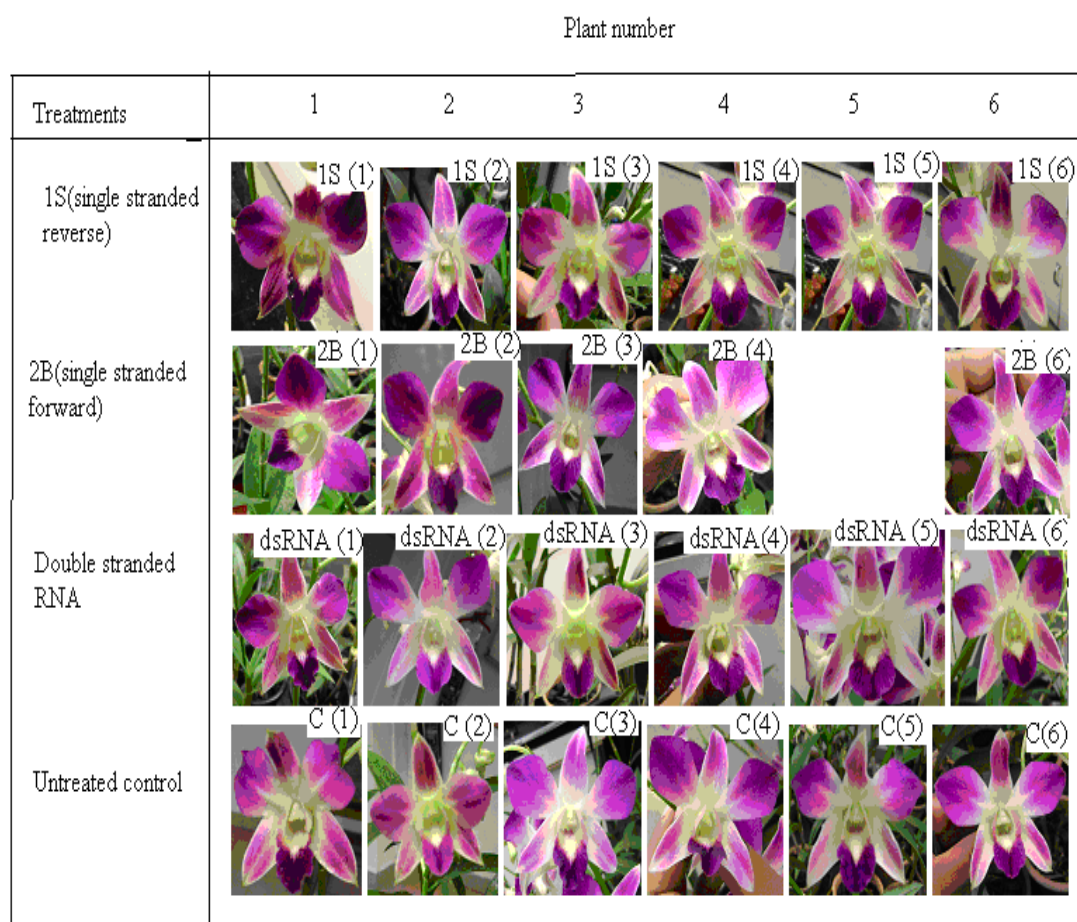


Figure 4.9: *Dendrobium Sonia* flowers from each treatment group showing no significant colour changes in the flowers

4.2.1 Semi quantitative RT-PCR to determine effect of RNA interference on *MYB9* gene expression in *Dendrobium Sonia*

Flowers from each treatment group that flowered within a day of each other were selected and these samples were pooled together for RNA extraction. A minimum of 3 flowers were selected from each treatment group. The sepals, petals and lip of each of these flowers were separated, pooled together and RNA was extracted from each. Three pooled samples of each flower tissue was tested using semi quantitative RT-PCR to determine *MYB9* expression levels. Flowers from buds treated that were larger in size were chosen and placed in one group while flowers from buds that were smaller in size when treated were placed in another group. Thus each treatment group was compared based on the type of

treatment and the size of buds when treated. A semi quantitative PCR was performed to determine if there was any effect of the different treatments on the *MYB9* gene expression in the flower samples.

As shown in figure 4.10, the sample 2Bb shows the *MYB9* gene expression is low in all three flower parts (sepals, petals and lip) compared to the other samples. The sample dsRNAb shows high expression levels of the *MYB9* gene in sepals, petals and lips. In samples 1Sa, 2Ba, 1Sb, 2Bb there was no expression of *MYB9* observed in the lip of the flowers; while dsRNAa, dsRNAb and Ca showed *MYB9* expression in all flowers parts i.e. sepals, petals and lips. The relative intensity of each band was calculated using the band densitometry software ImageJ. The average was taken for sepals, petals and lips to obtain average intensity and these values were analyzed in Microsoft excel to produce the graph in figure 4.11.

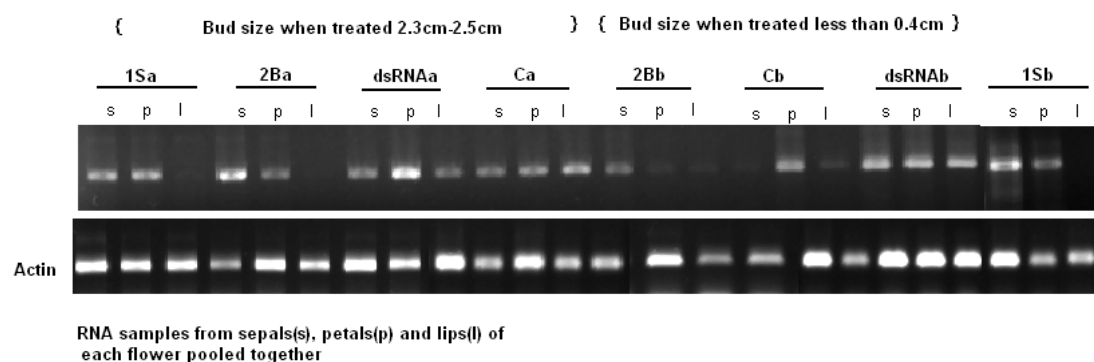


Figure 4.10: Comparing different treatments and their effect on *MYB9* gene expression

Lanes 1Sa, 1Sb: reverse ssRNA; Lanes 2Ba, 2Bb: forward ssRNA; Lanes dsRNAa, dsRNAb: (double stranded RNA); Lanes Ca, Cb: control.

s-sepals, **p**-petals, **l**-lip

a-bud size 2.3-2.5cm, **b**- bud size less than 0.4cm

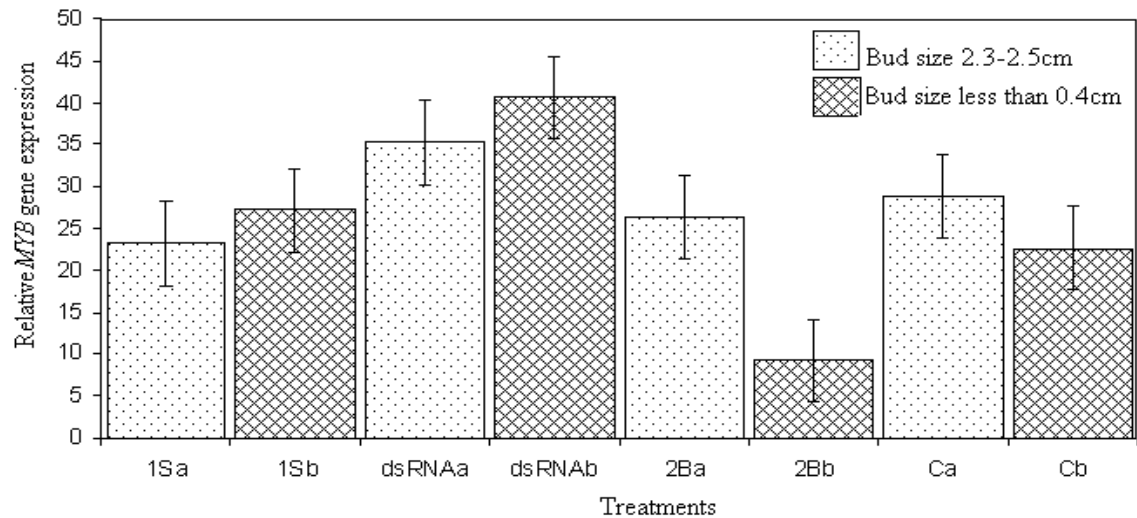


Figure 4.11: Relative *MYB9* expression for different treatments of crude bacterial extract on pooled flower samples (petals, sepals and lips combined)

1Sa and 1Sb: reverse ssRNA treatment; dsRNAa and dsRNAb: double stranded RNA treatment; 2Ba and 2Bb: forward ssRNA treatment; Ca and Cb: controls

A graph was made from the results in figure 4.10 by assigning values to each band based on the intensity with three samples per group. The values were averaged to obtain the *MYB9* expression levels for the pooled flower samples. The graph (figure 4.11) shows that samples treated with the forward strand ssRNA (2Ba and 2Bb) show relatively low *MYB9* gene expression. Those treated with dsRNA (dsRNAa and dsRNAb) showed higher *MYB9* expression compared to all the other samples. Sample 2Bb (bud less than 0.4cm long) showed lower expression of *MYB9* compared to 2Ba (bud 2.3-2.5cm long).

4.2.2 Semi quantitative RT-PCR to determine effect of RNA interference on *MYB9* gene expression in *Dendrobium* Burana Stripe

The treatments were repeated on a different *Dendrobium* variety, *Dendrobium* Burana Stripe. This variety produces flowers in which the sepals, petals and lips are all the same colour, thus making it easier to observe if there are any changes in colour expression in the flower (figure 4.12). First a gradient was performed to optimize RT-PCR conditions for

this orchid variety as shown in figure 4.13. A semi quantitative RT-PCR was done on these flowers as well to determine the effect of different treatments on expression of the *MYB9* gene (figure 4.14). The results showed no observable change in flower colour due to treatment with crude bacterial extracts as shown in figure 4.12.

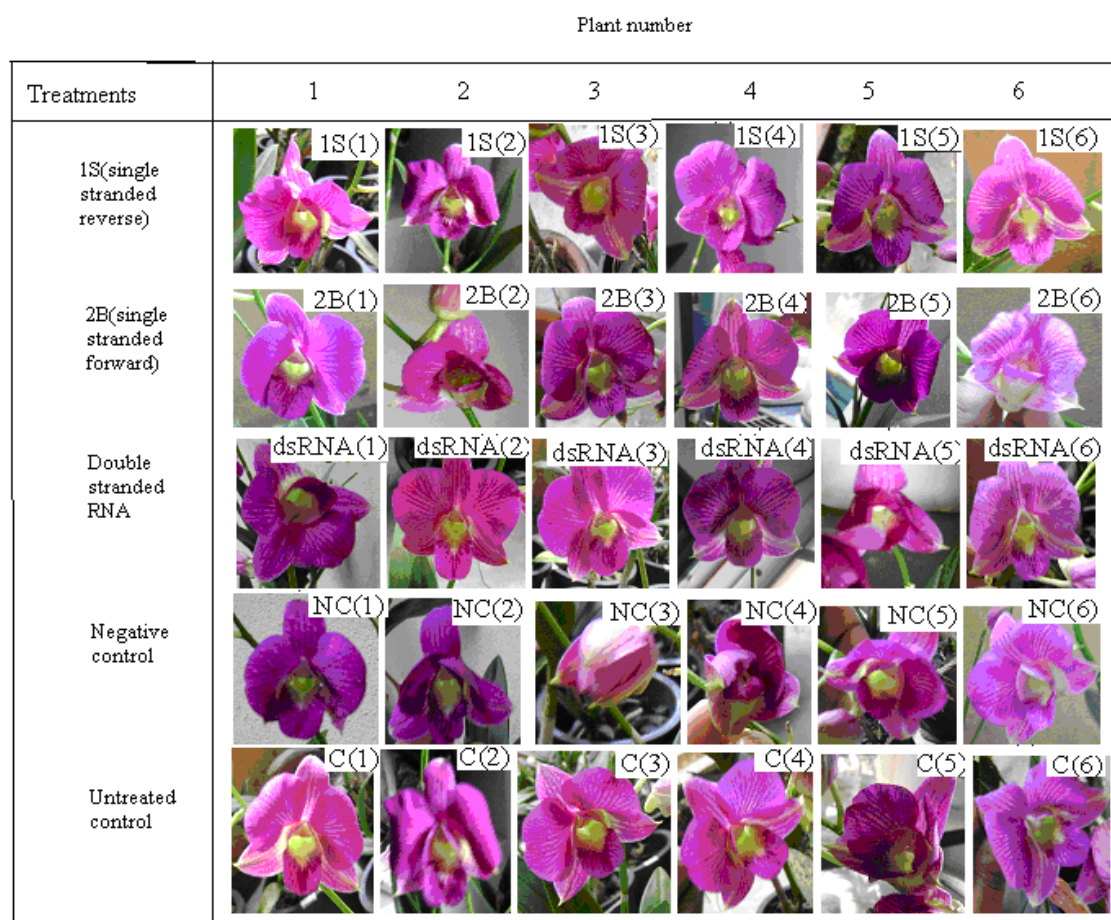


Figure 4.12: *Dendrobium* Burana Stripe flowers from each treatment group showing no significant colour changes in the flowers

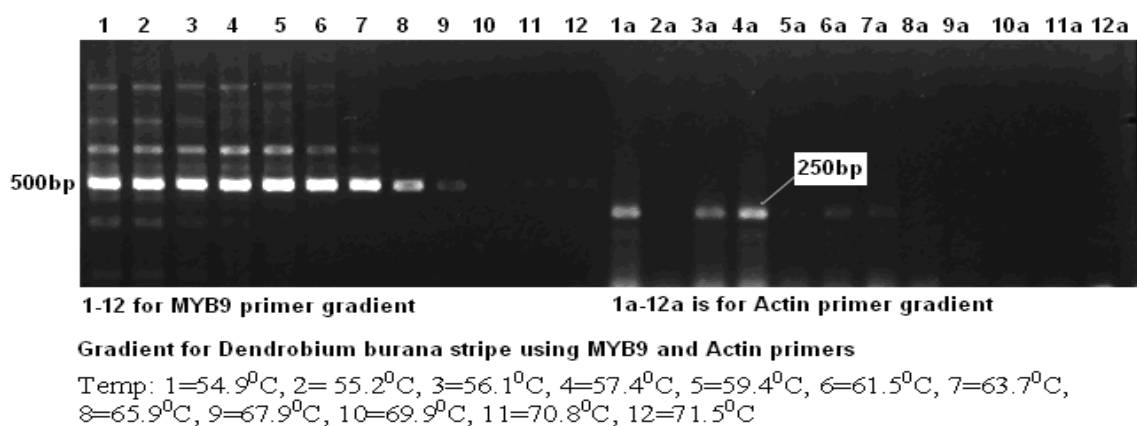


Figure 4.13: Gradient RT-PCR on *Dendrobium Burana* Stripe showing the optimum temperature range for *MYB9* primer is 63.7°C to 65.9°C and Actin primer is 54.9°C to 57.4°C

The semi quantitative RT-PCR was performed on whole flowers of *Dendrobium Burana* Stripe. The results are shown in figure 4.14 and 4.15. The band intensity values were obtained using the imageJ software and values were normalized for the actin standard. A minimum of five pooled flower samples were analyzed from each group to obtain intensity values which were used in Microsoft excel to generate the graph (figure 4.15). From the graph it was found that the relative *MYB9* gene expression in samples treated with dsRNA was slightly lower than that of all other treatments. The samples treated with 2B also showed lower expression of *MYB9*, while the untreated and negative controls showed slightly higher levels of *MYB9* expression.

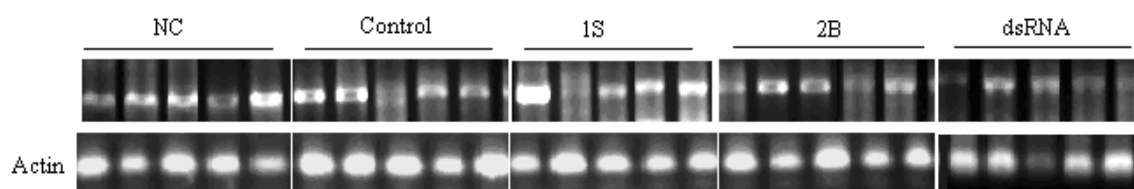


Figure 4.14: Effect of different treatments on *MYB9* gene expression in *Dendrobium Burana* Stripe

NC-negative control, Control-untreated control, 1S- reverse ssRNA, 2B- forward ssRNA, dsRNA- double stranded RNA

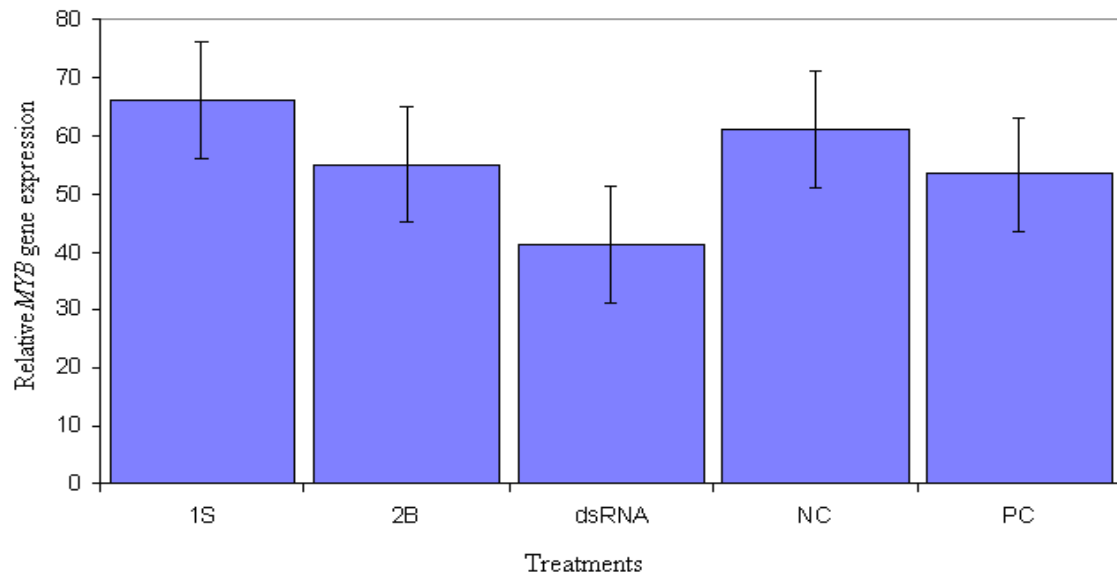


Figure 4.15: Relative *MYB9* expression for different treatments with crude bacterial extract on pooled flower samples

1S-reverse ssRNA, 2B-forward ssRNA, dsRNA-(double stranded RNA), NC- Negative control, PC-untreated control

4.2.3 M-Fold RNA secondary structure to determine stability of each treatment and effect on *MYB9* gene

To get some idea of the secondary structure of the *MYB9* ssRNA used to treat the orchid buds, the mfold program (Zucker, 2003) was used to analyze the RNA sequences for 1S reverse ssRNA and 2B forward ssRNA. The predicted secondary structures of the RNA are shown in figures 4.16 and 4.17. Some of the side branches are enlarged to show regions that are form stable dsRNA stems with higher Watson-crick base pairing and which could result in better gene silencing activity. Based on the ΔG values predicted by mfold, the forward ssRNA (2B) with ΔG of -208.07 was found to be a more stable structure than 1S reverse ssRNA (ΔG of -149.2).

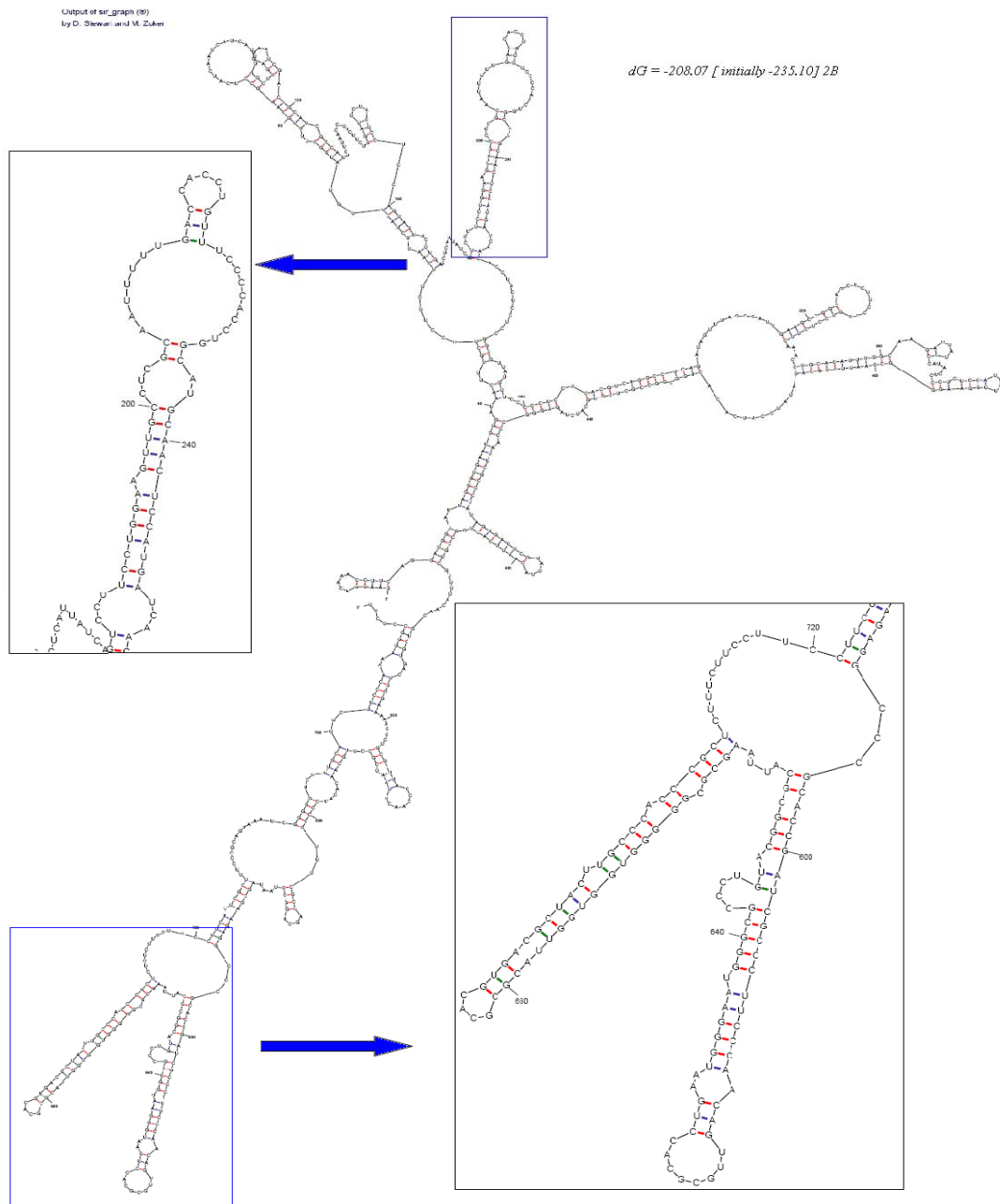


Figure 4.16: M-fold generated structure of the forward ssRNA (2B)

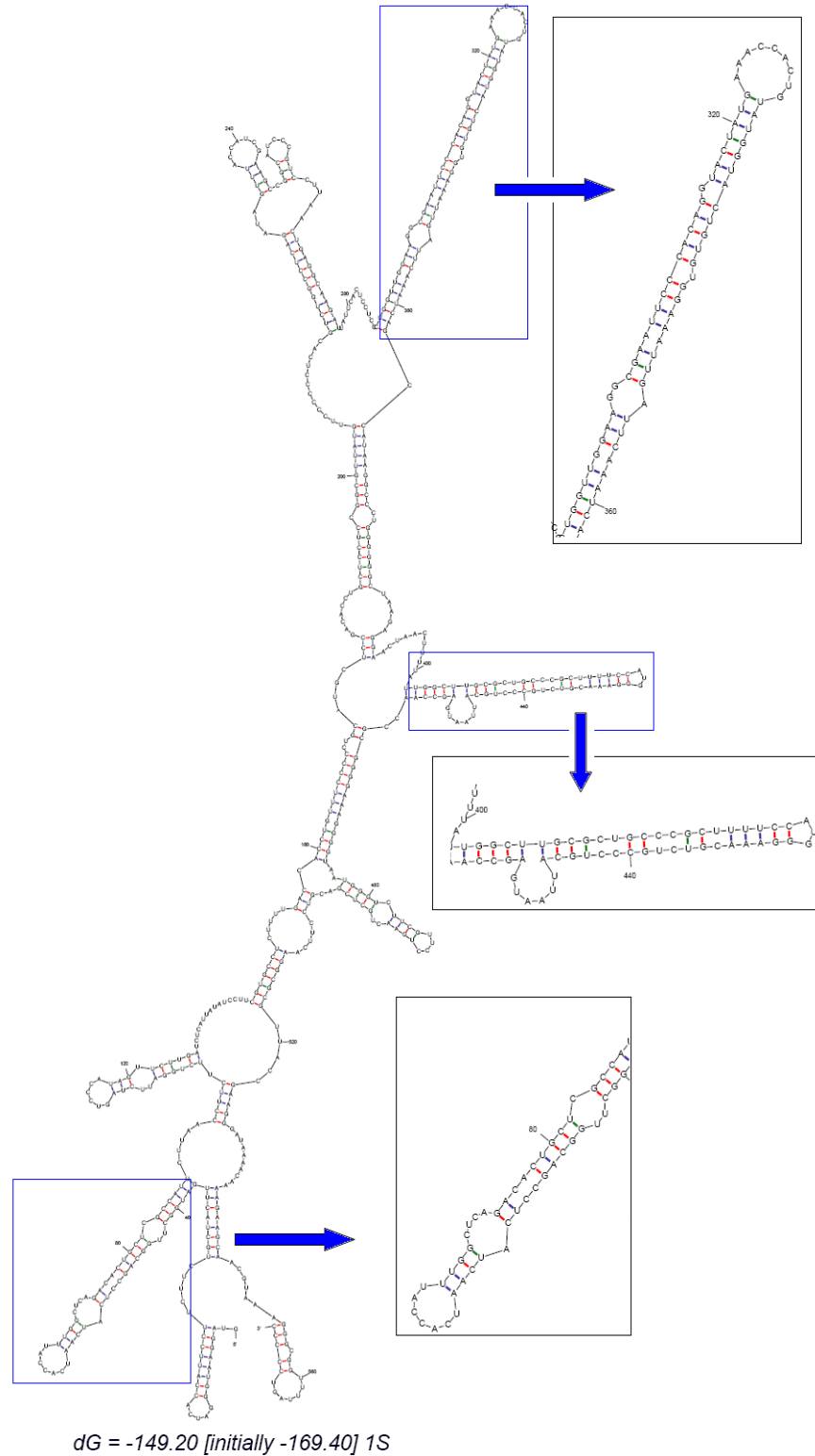


Figure 4.17: M-fold generated structure of the revers ssRNA (1S)

CHAPTER 5: DISCUSSION

5.0 Effect of crude bacterial extracts on *Dendrobium* Sonia flowers

In recent years the use of RNAi technology in gene silencing studies has become a widely used method to determine gene function in several organisms. Earlier studies showed the potential of this technique to knock down specific genes in plants and nematodes (Thackray, 2004). In this study RNA interference was carried out in the orchid *Dendrobium* Sonia to determine if *MYB9* gene expression in the flowers could be suppressed through exogenous application of homologous mRNA and if this would have any effect on colour production in *Dendrobium* Sonia flowers. The *MYB9* gene from *Dendrobium* Sonia was isolated and single stranded RNA was synthesized for both the forward and reverse strands to produce double stranded RNA that could be used in the RNA interference study.

The *MYB9* gene which was found in *Dendrobium* Woo Leng (*DwMYB9*) was targeted for this study due to its expression only in flowers and buds of the orchid suggesting its possible involvement in the phenylpropanoid pathway (Wu *et al*, 2003). Primers were designed for the *MYB9* gene using the *DwMYB9* sequence (Accession number: AF485900) (Wu *et al*, 2003). These primers were tested on *Dendrobium* Sonia and yielded a ~500bp fragment which was sequenced and analyzed in BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). This showed 98% similarity between *DwMYB9* and the *MYB9* gene from *Dendrobium* Sonia. The *MYB9* gene belongs to the *R2R3 MYB* gene family consisting of *R2* and *R3* repeats which are reported to be sufficient for sequence specific DNA binding (Wu *et al*, 2003; Jin and Martin, 1999). Once the *MYB9* gene sequence was successfully isolated and identified, it was cloned in order to isolate plasmids containing forward and reverse orientations of the *MYB9* gene. These were

transformed into HT115 strain of *E. coli* to produce dsRNA. This double stranded RNA was in the form of a crude bacterial extract that was applied to *Dendrobium* Sonia orchid buds with the abrasive cellite. The cellite served as a means to damage the outer cells of the orchid buds allowing the RNA to enter into the plant cells. As seen in previous studies crude bacterial extracts of dsRNA can be used to protect plants against virus infections. In studies conducted on *Nicotiana benthamiana*, crude bacterial extracts produced in HT115 *E. coli* containing dsRNA derived from viral sequences were applied to plant leaves using carborundum as an abrasive and it was found that this inhibited viral infection (Tenllado *et al* 2003). The bacterial extract purified with phenol and then applied to leaves resulted in plants that were free of viral infection up to 10 weeks post inoculation. Crude bacterial extracts prepared in a french press and sprayed with an atomizer on the surface of leaves also resulted in plants free of viral infection till the end of their life cycles.

The orchid buds were treated with the reverse single stranded RNA (1S) and forward single stranded RNA (2B) to determine if sense or antisense RNA had any effect on *MYB9* gene expression. The results showed no visible change in colour of *Dendrobium* Sonia flowers (Figure 4.11), however upon performing semi quantitative RT-PCR, the results suggested reduced *MYB9* expression in some flowers treated with forward and reverse ssRNA (Figure 4.12). The graph (Figure 4.13) showed the relative *MYB9* gene expression for different treatments with crude bacterial extracts. The dsRNA treatment showed a slightly higher level of *MYB9* expression than forward and reverse ssRNA and the control. Gene silencing of *MYB9* was most effective with sample 2B (forward ssRNA) on buds sized less than 0.4cm which showed the lowest level of *MYB9* expression. These results suggest that forward ssRNA suppressed expression of *MYB9* in 0.4cm buds while dsRNA treatments resulted in a slight increase of expression of *MYB9* in 0.4cm buds. For the other

groups, the standard errors were too large to make conclusive remarks and for all groups there were no visible changes in flower colour in the orchids. This could suggest that the *MYB9* gene is not involved in the phenylpropanoid pathway in *Dendrobium* Sonia orchids and may have another function that does not affect the phenotypic appearance of the flower, or that the changes in colour were too slight to be observed. Since certain *MYB* genes are known to be structurally similar but have different functions in different species or even within the same organism it is possible that the *MYB9* gene has a different role to play in *Dendrobium* Sonia flower development (Jin and Martin, 1999; Martin and Paz-Ares, 1997). It is also possible that the *MYB9* gene has a role to play in control or activation of certain steps in the phenylpropanoid pathway; however this may not directly affect production of anthocyanins in the orchid.

It is widely known that dsRNA induces sequence specific gene silencing and that the siRNA signal spreads along the mRNA target resulting in production of secondary siRNAs thus inducing transitive RNA silencing (Thackray, 2004; Lu *et al*, 2004; Agarwal *et al*, 2003). It has also been found that dsRNA can be as much as ten times more potent as a silencing trigger than sense or antisense RNA (Hannon, 2002), requiring only a few dsRNA molecules in the cell to trigger silencing (Thackray, 2004). Therefore it is possible that large quantities of dsRNA remained in the orchid cells resulting in the detection of higher values for *MYB9* expression levels. Other factors that could affect *MYB9* expression levels within the orchid include RNA stability, time between treatment and assay and amount of RNA in each treatment. Since the RNAi effect was still detected by semi-quantitative RT-PCR when the buds opened, it can be predicted that the effect was long lasting, about 2-3 weeks which is the time it took for most buds to open. In another study by (Tenllado *et al* 2003), it was also found that dsRNA was relatively stable when sprayed on leaves and

persisted in the plant several weeks after treatment. The amount of RNA in each treatment differed as well; therefore it is possible that higher RNA levels were detected for dsRNA because it was more stable and more RNA was present in the treatment.

MYB genes are involved in regulation of secondary metabolism in plants and thus have varied functions such as control of cellular morphogenesis, in signal transduction pathways responding to plant growth regulators, regulation of meristem formation and the cell cycle and phenylpropanoid metabolism (Jin and Martin, 1999; Martin and Paz-Ares, 1997). The *MYB9* gene may be involved in other aspects of flower development such as control of cell shape or in signal transduction pathways. The secondary folding structure of the forward and reverse ssRNA sequences was found using mfold (<http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi>), a bioinformatics tool that helps in prediction of RNA secondary folding structures. The results suggest that the forward ssRNA is able to fold into a more stable structure with more Watson-crick base pairs as compared to the reverse ssRNA (Figures 4.17 and 4.18). This prediction is consistent with the results of the RNAi study, as it could be expected that if the forward strand was more stable than the reverse it might have a better gene silencing effect on *MYB9*. The flower samples 2Ba and 2Bb were treated at different bud stages; 2Ba was larger between (2.5cm-2.3cm) while 2Bb was less than 0.4cm when treated. The expression of the *MYB9* gene was less in 2Bb indicating that RNA interference was more effective on smaller buds as compared to larger ones. However with the reverse strand, no significant changes were observed between samples 1Sa and 1Sb. The results also suggest that the silencing effect is prolonged lasting up to 2-4 weeks as the smaller buds were harvested only after opening (3-4 weeks after treatment) compared to the larger buds that opened (1-2 weeks after treatment). Even though a single construct was used in this experiment, the long dsRNA was expected to be cut up by dicer

enzyme to produce several siRNA that would bind to the mRNA and degrade it thus reducing expression of the gene (Milhavet *et al*, 2003; Vaucheret *et al*, 2001). However it is also possible to improve the experiment by constructing and testing more than one construct.

The fact that changes in *MYB9* expression were detected through semi quantitative RT-PCR indicates that the method of treatment was effective enough to introduce the dsRNA into the flower. However the extent to which the RNAi effect was spread through the flower seems to be random and unspecific. The treatment was applied on the surface of the buds which means that cells in the sepals would be the first to be exposed to the treatment, followed by petals and then the lip. In Figure 4.10 samples 1Sa, 2Ba, 1Sb and 2Bb show that *MYB9* was not expressed in the lip (l) but was shown to be expressed in the petals (p) and sepals(s). However the control sample and dsRNA showed *MYB9* expression in all three flower parts. Based on these observations it is possible that the RNAi effect did reach all parts of the flower since in samples 1S and 2B the *MYB9* gene was silenced in the lip and other flower parts. As shown in figure 4.9, some flowers looked lighter than others but there was no significant change in colour that corresponded to the treatments. In some flowers the sepals looked much lighter than in others; however *MYB9* expression from these flowers was similar and not affected by treatments indicating that differences in colour were due to natural variation as although the plants were produced *in vitro* they did not originate from the same parent plant. Usually most RNAi treatments involve independently scoring efficiencies for each individual sample and this gives a range of RNAi efficiency and a range of different phenotype from non-detectable to significant phenotypic differences. Often the efficiency of RNAi corresponds to the phenotype and can be grouped depending on the degree of the phenotype shown. Since in this study three

individual flowers of similar treatments were combined for further analysis this could also explain the random and unspecific results obtained.

Perhaps different or more efficient treatment methods could be applied to ensure proper and more even delivery of RNA into the orchid flower, e.g. injection into the plant or use of a virus to deliver the dsRNA. As seen in the nematode *C. elegans*, RNAi could be induced by feeding worms with HT115 *E. coli* expressing dsRNA corresponding to a specific gene or even through soaking worms in solutions containing dsRNA; however injection of dsRNA was the most efficient method of dsRNA introduction even though it is quite expensive (Kamath *et al*, 2000). VIGS(virus induced gene silencing) has also been used successfully in plants such as *Aquilegia vulgaris* and *Arabidopsis thaliana*, however it is more expensive and time consuming as *Agrobacterium*- mediated introduction of viral constructs is used. The results however show that it is an effective method of RNAi and in *A. vulgaris* it was successfully used to inhibit formation of purple wild type flower colour through silencing of anthocyanidine synthase (Gould and Kramer, 2007; Burch-Smith *et al*, 2006). The disadvantage with these methods is they are time consuming and relatively more expensive. Since using HT115 *E. coli* is an efficient and cost effective method of producing large quantities of dsRNA (Ongvarrasoponea *et al*, 2007), perhaps this method can be used to target genes that are directly involved in phenylpropanoid metabolism, such as chalcone synthase and this may be more successful for suppression of colour in flowers of the orchid. In principle, this method can be used for gene silencing as shown in studies where dsRNA was used to protect plants against viral infections (Tenllado *et al* 2003).

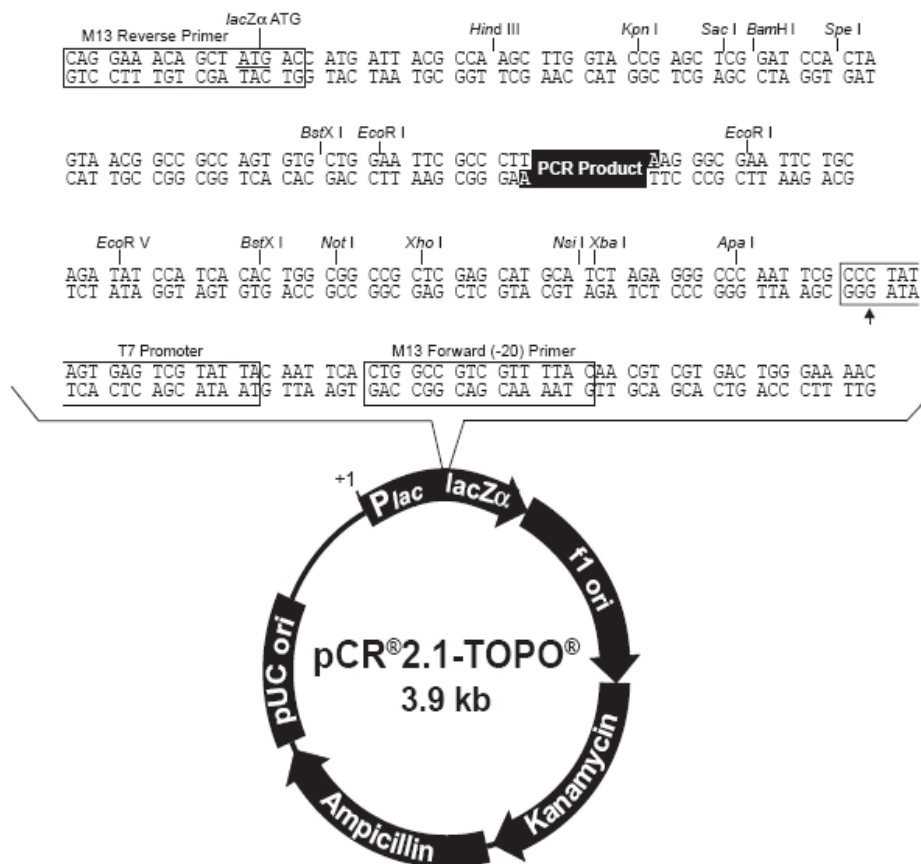
5.0.1 Effect of crude bacterial extracts on *Dendrobium* Burana Stripe flowers

Treatment with the same crude bacterial extracts on *Dendrobium* Burana Stripe showed different results compared to treatment of *Dendrobium* Sonia orchids. *Dendrobium* Burana Stripe was chosen for testing the treatments because it has flowers that are fully purple in colour thus any changes in flower colour could be observed more easily. Again the flowers showed no visible change in colour expression as shown in Figure 4.12. The semi quantitative RT-PCR results (Figure 4.14 and 4.15) showed there was some reduction in the expression of *MYB9* by dsRNA treatment in this variety of orchid. The treatment with the forward ssRNA also showed some reduction in the expression of *MYB9* transcript. The reverse strand (1S), untreated control (PC) and negative control (NC) showed higher *MYB9* expression indicating these treatments had no effect on *MYB9* expression. These results indicate that while there was some reduction of the *MYB9* transcript this does not have an observable effect on flower colour though it may control some part of the flower development process which was not detected. *MYB9* could be involved in other developmental pathways involving the cell cycle and perhaps influence time of flowering as seen with the gene *AtMYB65* which was found to be identical to *MYB9* in *Arabidopsis thaliana* (Colucci *et al*, 2002). Since the reduction in expression observed in this study was slight, this could be attributed to the particular gene sequences used in this experiment as usually more than one sequence is tested, therefore in future studies different parts of the sequence may be tested and this could give greater silencing and thus give clearer phenotype changes (Yan *et al*, 2006, Gould and Kramer, 2007).

CHAPTER 6: CONCLUSION

The main goal of this thesis was to investigate a fast, inexpensive and efficient biotechnological technique for study and manipulation of genes involved in the phenylpropanoid pathway in *Dendrobium* orchids. Previous studies have shown that RNAi holds great potential for regulating gene expression and thus has potential for altering gene expression in a sequence specific manner, thus could be used towards creation of new varieties of orchid and to investigate the function of genes involved in the phenylpropanoid pathway. The direct application of crude extracts of bacterial RNA containing *MYB9* gene sequences was able to show some reduction of transcript levels (or silencing) of *MYB9* in this study; however this had no clear effect on flower colour, indicating it may have been too weak an effect or that *MYB9* may have some other function in orchid flower development. Since this study has demonstrated that this method of RNAi can be applied to orchids for manipulation of transcription, further studies could be carried out targeting different genes involved in phenylpropanoid metabolism such as chalcone synthase. The method is relatively simple, inexpensive and rapid, in comparison with transformation methods, so should be suitable for studies involving slow growing plants such as orchid.

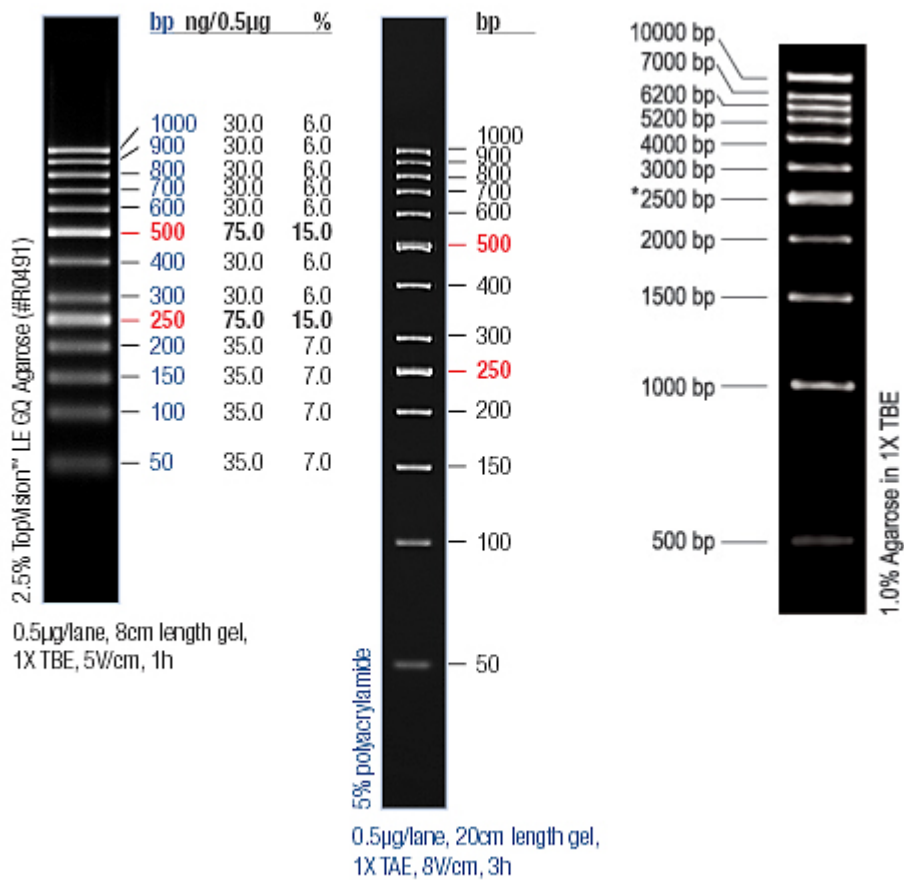
APPENDIX A: Plasmid map (pCR 2.1-TOPO) vector



Comments for pCR[®]2.1-TOPO[®] 3931 nucleotides

LacZα fragment: bases 1-547
 M13 reverse priming site: bases 205-221
 Multiple cloning site: bases 234-357
 T7 promoter/priming site: bases 364-383
 M13 Forward (-20) priming site: bases 391-406
 f1 origin: bases 548-985
 Kanamycin resistance ORF: bases 1319-2113
 Ampicillin resistance ORF: bases 2131-2991
 pUC origin: bases 3136-3809

APPENDIX B: Agarose gel markers



Restriction enzyme table for sample 2B generated in BioEdit to determine possible restriction enzyme sites on the fragment The enzymes highlighted in the boxes (Table 4.1) were selected for testing on the plasmid samples because they had one or two restriction sites present on the plasmid and also as they were available.

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7.0 REFERENCES

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